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GENERAL CYTOLOGY

A TEXTBOOK OF CELLULAR STRUCTURE AND FUNCTION FOR STUDENTS OF BIOLOGY AND MEDICINE

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PREFACE

During the summer of 1922 several meetings were held at the Marine Biological Laboratory at Woods Hole, Massachusetts, in order to consider the advisability of making a co-operative attempt to state in general terms what is known or conjectured regarding the principles which govern cellular structure and function; or, in other words, to present briefly for the first time within the scope of a single volume data concerning the cell fundamental, alike, to the sciences of botany, zoölogy, physiology, and pathology. It was recognized that this would involve a close rapprochement between physicochemical and morphological points of view, which in itself would be beneficial. It was decided that the best plan would be to emphasize the results obtained in different lines of work bearing upon the cell, as the fundamental unit in health and disease, mentioning methods of investigation only when necessary and historical data only in order to place the results in their proper perspective in the development of the subject; but to supply copious references to the current literature so that any particular line of thought could be followed up systematically and at a minimum expense of time and effort; this information to be given in such a form that it would be useful not only for students but also for investigators. Upon careful consideration it was found that a presentation of this kind naturally fell into subdivisions, every one of which could profitably be treated by workers in the laboratory, each one of whom was accordingly invited to assume full responsibility for a portion of the book. In this way the labor involved was shared and did not fall heavily on the shoulders of any single individual. The unique opportunity thus afforded for friendly and informal consultation between the different contributors greatly facilitated the enterprise. Indeed, it is doubtful whether the object in view could have been approached if the individuals concerned had been widely scattered in different parts of the country. Under these circumstances, and in consideration of the fact that several of the contributors had developed their lines of study by availing themselves year after year of the facilities for investigation offered at Woods Hole, the volume, as it stands, is to be considered, to some extent at least, as a contribution from the Marine Biological Laboratory.

E. V. COWDRY

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SECTION I

INTRODUCTION

By
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INTRODUCTION

EDMUND B. WILSON

The word cytology denotes the study of cells, and in its widest sense this study might be said to extend into every branch of biology that is directly concerned with cell phenomena. In practice, however, cytologists have not made for their subject so ambitious a claim. They have commonly employed the word in a much narrower sense to denote the microscopical study of cells with especial reference to their structure and structural transformations. As thus limited cytology, evidently, is nearly related to the older histology, a subject treated as a branch of anatomy dealing with the structure of the tissues (*Gewebelehre*), and often designated as microscopical anatomy. Such a restriction of the subject, however, would no longer be possible even were it desirable; for cytology long since outgrew the limits of merely morphological inquiry. In respect to its relation to histology, we are struck by the fact that the earlier investigators of the cell did not clearly distinguish between the two subjects.[1] Cytology first received recognition as a distinct subject when later researches served more and more to focus attention upon *the individual cell considered as an organic unit.* Even now the boundary line between cytology and histology cannot, and need not, be very strictly drawn. It stands for no more than convenient practical usage.

That the cell may be treated as an elementary organism or organic unit was indicated in a general way by Schwann, Virchow, and other earlier observers; and some of these, in particular Brücke (1861), insisted that cells must possess some kind of structure or organization more complex by far than any made visible by the microscopes of the time. Cytology as such first arose, however, when the internal organization of the cell was made more accessible to investigation by improvements in microscopical technique and by the discovery of more favorable objects for study. Its beginnings are found in a remarkable series of researches on the fertilization and cleavage of the animal egg which began in the early seventies, more than thirty years after promulgation of the cell theory by Schleiden and Schwann (1838–39); and the leaders of this movement were for the most part students of development rather than of histology. Cytology, therefore, may be said to have taken its immediate point of departure from embryology; and its close association with histology in the narrower sense first took place with the demonstration that the apparatus of cell division in the tissue cells is in all essential respects identical with that observed in the ovum and the blastomeres into which it splits up during the earliest stages of development. The interest of these pioneer researches centered in the discovery of indirect cell division or *karyokinesis* (Schleicher), later known as *mitosis* (Flemming), a process involving complexities wholly unsuspected by earlier observers; and down to our own day these phenomena, together with the closely related ones displayed in the fertilization of the ovum, have continued to hold a central position of importance because of their fundamental significance for the principles of genetic continuity in living organisms. In the seventies these phenomena were wholly new; and the rich horizon of discovery thus opened claimed for a time the almost exclusive attention of the earlier cytologists.

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[1] The distinction is not recognized in Haeckel's *Generelle Morphologie* (1866) but is clearly drawn in Carnoy's *Biologie Cellulaire* (1884).

It may seem strange that the subject should so long have been dominated by morphological studies, especially on fixed and stained cells, when we recall those illuminating researches on living cells by Dujardin, Max Schultze, DeBary, Kühne, and other pioneers, which led to a general recognition of protoplasm as the physical basis of life. The explanation lies in part in the failure of the earlier microscopes to make visible in living cells an organization in any degree adequate to explain the vital activities, while the fixation of cells by certain coagulating agents, such as dilute acetic, osmic, or chromic acids, often makes visible a definite and complex structure brought still more clearly into view by the use of certain dyestuffs, such as carmine or hematoxylin. The use of these methods gained headway with the rapid improvement that took place during the seventies and eighties in high-power microscopical lenses and in technical methods for the preparation of fixed and stained sections. Such methods have played an indispensable part in the advance of modern cytology; without them, indeed, many of its most fundamental discoveries would have been long deferred or impossible, for example, the formation and splitting of the spireme threads or the history of the central boides in mitosis. These methods, nevertheless, involve many possible sources of error; and although in theory this has always been held clearly in view, in practice even skilled observers have not always found their way safely amid what Michael Foster called the "pitfalls of carmine and Canada balsam." To this source are traceable some important errors in the earlier work; but their effect was less serious than was at one time feared, and they did not invalidate the main discoveries of the foundation period of cytology. Nor were studies on living cells wholly neglected during this period. Far from this, some of the most important researches of the seventies were in fact largely carried out by such studies. The importance of the study of coagulation phenomena and of critical comparison of the conditions observed in fixed (coagulated) and stained cells with those pre-existing in the living object was to a certain extent recognized from the beginning; but it was only later that this side of the subject received the attention that it deserved and that the study of living cells once more came to the front. Cell research then entered upon a phase of new and broader activity in the course of which cell morphology and cell physiology tended more and more to amalgamate. One important result of this was to overthrow certain of the earlier conceptions of protoplasmic structure, such as the reticular theory, that had been based on the study of fixed material. Another was to arouse a salutary skepticism concerning the so-called "microchemical" methods in so far as the staining reactions of the cell components had been employed as a guide to their nature and origin. For certain purposes such methods are indispensable; but unless used with due caution they may be a prolific source of error, as the history of the subject abundantly demonstrates. Out of all this grew a more rational treatment of the whole subject, and a gradual affiliation of cytological methods of the earlier type with those of the physiologist, the physicist, and the biochemist. How this came to pass will be made clearer by a brief historical survey.

1. The history of cell research since the promulgation of the cell theory may conveniently be divided into three periods, the first beginning approximately in 1840, the second in 1870, and the third in 1900.[1] The first of these includes the early development of the cell theory and the second that of modern cytology and cellular embryology. In the course of these two periods our general conceptions concerning the mechanism of development and heredity began to take on definite form. The third period, opening with the rediscovery of the Mendelian phenomena of heredity, includes the detailed genetic and cytological analysis of these phenomena; the minute cytological analysis of nucleus and cytosome; and the modern experimental study of cell physiology. These periods are separated by no sharply marked lines of demarcation; they are distinguished merely as a matter of convenience. Without attempting to review them in detail we may briefly indicate a few of the outstanding results which they have brought forth.

The conception of the multicellular organism as an assemblage of elementary units or cells was clearly indicated by Schwann and was set forth in a masterly manner by Virchow (1855–58) who emphasized especially its physiological aspects and thereby prepared the way for a revolution both in physiology and pathology. The discoveries of the first three decades following Schleiden and Schwann brought forth two general results of fundamental importance. One was the demonstration that cells arise only by division of pre-existing cells, a result involving the still broader conclusion that cell division constitutes the fundamental basis of the law of genetic continuity in the living world. This conception was clearly set forth in its full significance between 1850 and 1860 by Remak and Virchow, though many others contributed to it. Equally momentous was the demonstration that protoplasm, i.e., the substance of cytosome and nucleus, constitutes the physical basis of life. This conclusion, gradually emerging from the work of

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[1] This division of the subject is taken from the forthcoming third edition of my general work on *The Cell in Development and Inheritance.*

Max Schultze, DeBary, Cohn, and their fellow-workers (1860–70), first placed the study of the cell on a secure basis and prepared the way for all that followed. To the same period belong the earliest attempts at a logical analysis and classification of the morphological components of the cell system, prominent among them being the conclusions of Lionel Beale (1861 and later) who first drew a clear distinction between a primary living "bioplasm" or "germinal matter" and the "formed materials" of the cell conceived as secondary, and in many cases lifeless, products of the living stuff. During the whole of this period, however, only the most rudimentary notions were arrived at concerning the finer structure of cells, while the complex internal operations of cell division, maturation, fertilization, and the associated phenomena remained totally unknown.

2. The second period began in the early seventies with the epoch-making researches on fertilization and cell division, above referred to, which laid the foundation of modern cytology. The leaders in this movement were Auerbach, Schneider, Fol, O. Hertwig, Bütschli, Van Beneden, Flemming, and Strasburger, whose discoveries gave the point of departure for all later investigations in this field. On the physiological side, an outstanding event was the appearance of Claude Bernard's celebrated work entitled *Leçons sur les Phénomènes de la Vie* (1878–85), in which first appears a clear recognition of the close relations between chemical and morphological synthesis, together with a precise formulation of the view that the nucleus plays a leading rôle in the synthetic processes. This work, though apparently lying in a widely different field, logically belongs with the efforts of cytologists and theoretical writers during the decade between 1880 and 1890 to identify and to analyze the physical basis of heredity.

Among the most significant of these efforts were the cytological works of Van Beneden, O. Hertwig, Strasburger, and Boveri, and the theoretical writings especially of Roux, Nägeli, and Weismann. Nägeli (1884) first directed attention specifically to the possibility that heredity may be effected by the transmission of a specific *idioplasm* or germ plasm. Roux (1883) pointed out the fundamental significance of mitosis as a mechanism by which specific "qualities" borne by the nucleus may be transmitted and distributed in a particular manner. Van Beneden's great work on the fertilization and cleavage of the parasitic nematode *Ascaris megalocephala* (1884, 1887), followed by the more detailed cytological studies of Boveri (1887–90 and later), brought forth numerous results of far-reaching interest, which first served to direct attention to the possible importance of the chromosomes in heredity; while O. Hertwig and Strasburger (1884) simultaneously reached the conclusion, based on the phenomena of fertilization in both animals and plants, that Nägeli's idioplasm is identical with the nuclear substance. The widely read essays of Weismann on heredity and its mechanism, published between 1881 and 1888, set forth the broader bearings of cytology in relation to heredity and development, while his important general work on *The Germ Plasm*, published in 1892, brought forward an interesting speculative attempt to analyze the architecture of the idioplasm and the mechanism of development. Some of Weismann's conclusions were subsequently overthrown; nevertheless his illuminating discussions contributed in an important way to the spread of interest in these problems, and to the general development of our conceptions of heredity.

The decade from 1890 to 1900 was a period of steady expansion, which prepared the way for a new outburst of cytological activity. At this time the widespread interest in studies on the nucleus in relation to heredity somewhat overshadowed those relating to the cytosome; nevertheless investigations on the structure and structural transformations of the cytoplasm and its components were steadily advancing. Attempts to formulate a general conception of protoplasmic (i.e., cytoplasmic) structure had previously been made by several prominent investigators. Among the most familiar was the conception of protoplasm as a netlike or reticular structure (Leydig, Klein, Heitzmann, Van Beneden, Carnoy) or as consisting of separate fibrillae suspended in a transparent ground substance (Flemming, Heidenhain, Ballowitz). These particular views were unduly influenced by the study of fixed or coagulated cells; and the reticular theory in particular has proved extremely improbable, if not wholly untenable. On the other hand, more modern studies have constantly added weight to the view of the earlier investigators that living protoplasm considered as a whole has the properties of a more or less viscid liquid, and constitutes what is now generally designated as a colloidal system. The foremost place among the supporters of this view must be given to Bütschli, who from the beginning steadfastly held to the conclusion that the most usual, if not the universal, structure of living protoplasm is an alveolar or emulsion-like formation, and that the netlike formation, so often seen in fixed protoplasm, is either an optical illusion or a coagulation artifact. During the eighties, important progress in the study of coagulation phenomena with reference to the structure of protoplasm as seen in fixed material was made by Flemming, Berthold, Schwarz, and others, while in the nineties the subject was more critically examined especially by Bütschli (1892, 1898), Fischer (1899), and Hardy (1899).

Meanwhile critical study of the formed components of protoplasm was steadily advancing; and here belong numerous beautiful researches on the finer structure and histogenesis of the various types of tissue cells and of the germ cells, in both animals and plants. Of especial interest in their broader bearings were the researches of A. Meyer and of Schimper (1883–85) on the plastids of plant cells; those of Van Beneden (1883–84) and Boveri (1887–90) on the central bodies of animal cells; and those of Altmann (1886–87, 1894, etc.) on the general significance of the cytoplasmic granules. This latter question, discussed by many earlier observers from the time of Henle (1841) onward, was set forth in a remarkable manner by Altmann; and while his conclusions were too strongly colored by theoretical considerations, they have exerted an important influence on the study of the cytoplasmic structures in recent years. Of great interest in this direction have been researches on the chondriosomes under the leadership of Benda and Meves, and on the Golgi apparatus by Golgi, Cajal, and many others, which have opened many new and fundamental questions concerning origin and transformations of cytoplasmic cell components generally.

Many other important new lines of cell research were opened during this period. One was the foundation of cellular embryology, or cell lineage, through the work especially of C. O. Whitman on the development of leeches (1878), of Rabl on that of gastropods (1879), and of Van Beneden and Julin on ascidians (1884). These researches, a sequel to the earlier ones of Kölliker (1844) and of Remak (1850–55) on the cleavage of the ovum, sought to trace the parts of the embryo back not merely to the germ layers but to the individual blastomeres from which they arise, and ultimately to corresponding regions of the unsegmented egg. Studies in this direction, carried out in much detail in later years, made known facts of fundamental importance for an understanding of the general mechanism of development, and formed an indispensable adjunct to an experimental analysis of the phenomena set on foot by Pflüger, Roux, and Chabry, between 1883 and 1890, and carried forward by Driesch, Boveri, and many others during the following decade. To the same period belongs the initial experimental work on the chemical environment of the egg and early embryo by O. and R. Hertwig (1886–87), which stands among the earliest studies in experimental cytology. The beginnings of experimental cell physiology belong to an earlier period; but a powerful impulse to its further development was given by the work of the Hertwigs, and by the experimental studies along similar lines between 1890 and 1900 of Boveri, Driesch, Herbst, R. Hertwig, Morgan, and Loeb, which culminated in the discovery of artificial parthenogenesis (1899).

3. The third period of cell research coincides with the new era in genetics that opened in 1900 with the rediscovery of the Mendelian phenomena of heredity by DeVries, Correns, and Czermak. This discovery was the outcome of purely genetic experiments on hybrids; but almost at the moment of its announcement, by a remarkable coincidence, cytologists had independently arrived at a point where the cytological basis of the phenomena could be clearly recognized. Rückert eight years earlier (1892) had briefly suggested a conjugation and disjunction of corresponding paternal and maternal chromosomes in meiosis and an exchange of material between them ("amphimixis of the chromosomes"), thus to a certain extent foreshadowing the modern explanation of the Mendelian segregation and of recombination by "crossing-over." Montgomery (1901), without knowledge of Mendel's fundamental law of segregation, brought together almost all of the essential data for its explanation,

though he did not bring them into specific relation with the genetic phenomena. He pointed out the constant size differences of the chromosomes; emphasized the presence in the diploid groups of paternal and maternal homologues in pairs, and accepted the conjugation of these homologues in synapsis, and their disjunction in the reduction division. Boveri, in his remarkable paper on multipolar mitosis (1902) demonstrated experimentally the determinative action of the chromosomes in development and proved their qualitative differences in this respect. A possible connection between the Mendelian disjunction and the reduction division was suggested nearly at the same time by several observers, including Strasburger, Correns, Guyer, and Cannon. It was, however, Sutton (1902–3) who first clearly set forth in all its significance the cytological explanation of the Mendelian phenomena that is offered by the behavior of the chromosomes, and thus initiated the remarkable movement in this direction that followed.

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An outstanding feature of this movement has been the close co-operation between cytology and genetics in the critical examination of heredity. Conspicuous examples of this are offered by the modern analysis of sex and sex-linked characters, of linkage and recombination phenomena, of non-disjunction and its many consequences, and of the special types of genetic behavior shown by a great variety of mutants and hybrids. So effective has this treatment of heredity become as a practical means of analysis that genetic results may often most conveniently be stated in terms of the chromosomes, even in cases where the underlying cytological phenomena have not yet been observed or are presumably of such a nature as to preclude the possibility of direct microscopical analysis. In many such cases predictions concerning the cytological conditions have been verified in the most striking manner; in others the genetic results are still far in advance of the cytological (e.g., in case of the chiasmatype theory of crossing-over or the chromosome interpretation of gynandromorphism); but it is an impressive fact that among the numerous cases in which both genetic and cytological evidence are available not one offers an actual contradiction between the genetic data and the cytological. The chromosome theory of Mendelian heredity has thus become a working theory of the first rank, and one that offers every prospect of future development; while the more general questions that it has raised concerning the organization of the nucleus and of the cell as a whole stand among the most interesting problems of modern biology.

A second prominent feature of the present period has been the steady growth of interest in the cytoplasmic system and its formed components, especially the chondriosomes and the Golgi bodies, and their possible relation on the one hand to the processes of histogenesis, on the other to the fundamental organization of the cell. A leading part in this movement has been played by improvements in cytological technique which have made possible the more certain identification of these cell components and their transformations.

The numerous questions that have here been raised are still in a rather unsettled state, and researches in this field are now in the full tide of progress. The fundamental interest of these questions for our general conception of the cell system is emphasized by studies in experimental embryology, which have demonstrated the importance of the cytoplasm in the specification of cells and the general determination of development. The field here opened has as yet hardly been explored; but it has become clear that we cannot hope to arrive at any understanding of the mechanisms of localization and histogenesis without extended further studies in this direction.

In close connection with the foregoing has been the rapid development since 1900 of observation and experiment on living cells. Experimental studies in this field have in part made use of purely mechanical or physical methods, such as the displacement of the cell components by the centrifuge or by mechanical pressure, and the actual dissection of cells under the microscope. An important advance in this direction has been the development since 1910 of the so-called microdissection apparatus by Barber, Kite, and Chambers, which makes possible surgical operations on living cells under high-power lenses by the use of mechanically controlled fine glass needles or capillary tubes. Studies along these lines have steadily tended to emphasize the conception of the cell as a colloidal system and have led to many interesting modern attempts to imitate or model certain of the cell activities in artificial systems, as was long ago undertaken by Bütschli. In connection with studies of this general type may also be mentioned the study of isolated living cells and tissues, initiated by the protozoölogists and embryologists of an earlier period and carried much farther by the more recent studies of Harrison and his successors on the continued growth and differentiation of isolated tissue cells *in vitro* for long periods of time.

Of the first importance, finally, have been studies on the effect on living cells of changed conditions in the physical or chemical environment; and it is here, especially, that cytology passes over into cell physiology. A rallying-point for the more modern work in this field was given by the studies which culminated in the discovery of artificial parthenogenesis, and from them have branched out numerous lines of investigation, many of which stand today in the foreground of biological interest.

We cannot here attempt to follow in detail the general history of these various lines of inquiry. We have briefly indicated how the earlier morphological cytology has broadened out into a many-sided *cellular biology* (to employ the phrase of Carnoy) in which observation and experiment, morphology and physiology, have entered into close affiliation with one another and with biophysics and biochemistry. The result has been to create a new cytology, a new cell physiology, a new cellular embryology, and a new genetics; and these various lines of inquiry have now become so closely interwoven that they can hardly be disentangled. This much-to-be-desired result has been made possible by an always growing co-operation between lines of attack widely different in method and seemingly in point of view. Such concerted effort in cell research long seemed an almost unattainable ideal; but its realization now seems close at hand. The present book has been undertaken in hope of furthering this co-operation. In the nature of the case it is hardly possible to arrive at complete unity in a work produced by several collaborators representing widely diverse fields of research. Such a group, however, can at least bring to their task a broader and more critical knowledge of the subject than any single writer can at this day hope to command.

SECTION II

SOME GENERAL ASPECTS OF THE CHEMISTRY OF CELLS

By

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SOME GENERAL ASPECTS OF THE CHEMISTRY OF CELLS

ALBERT P. MATHEWS

I. CHEMISTRY AND PSYCHISM

In this section on the chemistry of the cell we shall proceed at once to the discussion of those fundamental chemical processes which are at the bottom of all cell life of every kind, both plant and animal—processes of which hitherto there has been no explanation, but which the advance of chemistry and physics has now made it possible for the first time to formulate in a clear manner.

The discovery that atoms consist of a number of electric charges, positive and negative; that every oxidation is an electric current, and every electric current an oxidation and reduction; the refinement and clarification of ideas of energy and its transfer from molecule to molecule and from atom to atom in definite units or quanta, with resulting change in reactivity, instability, power of combination, and in the configuration of the molecules and the very atoms; the increasing precision of conceptions of chemical affinity—all of these have vastly aided the biochemist to make some approach to a solution of the problem of cell life. For the first time he is able to see how the living machine may be acting, and while his conceptions are no doubt still hazy and indefinite where he desires to be definite, yet they are vastly clearer than they were, and it is his duty to apply every new physical and chemical conception to the solution of this absorbing problem. And these new conceptions help him immensely. He sees that life and electricity are indissolubly associated, since every atom is a system of electrons; every transfer of energy, and energy itself, is but so much electric current and magnetic flux. The body appears to him essentially as a battery, with a series of resistances and condensers, made up of conductors and dielectrics. The water, the salts, the graphite, i.e., the carbon compounds, of which it is composed make the living battery. Rignano has even gone so far as to liken the storage of memories to the storage of potential electricity in a storage battery; and Crile (1923) has likened the brain to a self-charging condenser.

The biochemist, indeed, has been transformed into an electrical engineer, but an electrical engineer in embryo, in process of becoming; for he is not yet able to understand completely the battery which is put in his charge. He cannot yet construct even the simplest of these; he cannot set one battery and the motor attached to it running or even stop one already running in such a manner that he can set it going again, although he may slow it almost to stopping and increase the speed once more; indeed, he can do no more as yet than see that water and proper chemicals are put in the battery and that the bearings of the motor are oiled and that it is kept reasonably clean. He is rather a cleaner and oiler than the engineer. He is still in the apprentice period of his career. But guided by the great Engineer, Chemical, Electrical, and Mechanical in one, who planned the machine, he hopes some day to make repairs necessary to keep it going for a longer period, and ultimately to make similar machines of a simple kind himself.

But even when we have a reasonably clear picture of these physical things, we cannot make a complete explanation of the chemistry of the cell until we know another and equally important factor which is at present wholly neglected by the chemist and physicist, namely, the psychic element which is the most characteristic, indeed, one might say *the* characteristic thing in living organisms. For living organisms are the largest, as they are the only, psychic units yet recognized. Living things show an attribute which we may call mentality or psychism, and this psychism is as yet unrecognized elsewhere than in living things. No one speaks of the psychology of this great rock upon the illuminated surface of which we crawl, our mother-earth; no one, that is, but the poets, those inspired seers of truth, who catch a glimpse through the fog of the great mountain peaks ahead of us. But who can deny to the inorganic earth that which is in the same inorganic elements when in the organized, the organic form? The biochemist of the future then must be more than an electrical engineer, for he must be poet and psychologist as well.

The psychologist of the future will discuss the psychology of hydrogen, of oxygen, indeed that of the electrons, positive and negative, themselves. For who can doubt that those properties of the atoms which show themselves in the psychical phenomena of living things are also present in the same atoms in the inorganic form? For the atoms are the same in living and lifeless, and every moment they are turning from the one to the other. As Du Bois Reymond put it, the atoms of iron in the great driving wheel of the locomotive and in the brain of the poet are the same.

We cannot understand chemistry, therefore, and certainly not biochemistry, the chemistry of cells, until the relation between material and psychic things is worked out. We must know what is that psychism always associated with all living matter and presumably with all lifeless also. What is the relation between the psychic and the material? Do the two interact? Is it possible to influence the psychical processes in our brains by radiant energy? Can mind act on mind directly through this ether which connects and penetrates both? Can we have knowledge other than through the senses? Our own psychical processes clearly depend upon the oxidations going on in the brain. When the oxidation is low, so is mentality low; when it is high, so is mentality high. We can then effect the psychical process by slowing or increasing the chemical rate of reaction. Is the converse of this also true? Can the increase in psychism, in psychic potential, bring about a change in the rate of oxidation? This as will be seen is a most fundamental question. We must not close our minds, but hold them open and look keenly at this great problem, this great unexplored mountain, looming so fortunately on the horizon before our successors; for it holds out to the generations yet to come great possibilities for increase of knowledge. From it may be secured, we hope, a glimpse into that promised land toward which we are journeying.

We have found that matter is not continuous; that it exists in a particulate form; in the form of units: of atoms and molecules; and finally of electricity, the electrons. And energy, too, is particulate. It is not radiated uniformly, but in units which are the product of energy time and frequency, called quanta. There is reason for believing that the ether itself either already consists of definite units, or has the property of crystallizing as it were into such units at the proper touch, those units when irreversible and minute being the electrons; and when larger and reversible, making a ray of light. Light is perhaps nothing else than a progressive and partial and reversible formation of large electrons out of the ether. So perhaps it may be also with psychism; and we shall find in the future that it, too, is particulate and made up of units of definite amount of psychism which we may call psychons. We may go even farther and say that the ether itself—continuous substratum capable of becoming granular—is psychism, as it is also potentially matter, and that when the two electrons, positive and negative, arise as irreversible material units within it, so also irreversible units of psychism arise at the same time. The great Being—Time and Space, Infinity and Eternity—thus sets aside from itself these irreversible minute portions which become animals and plants, men and women, planets and suns, electrons and atoms. And if, as is possible, in every beam of light there is an almost instantaneous and *reversible* formation of such electrical and psychical elements, light would have also its psychical side, and become a propagated psychical disturbance. And light is the great creative agency which has created all matter and life itself. To transform the ether, the very body of the Infinite, into man, matter, and stars—light, i.e., thought, has been the creative agent.

Hence the biochemist, looking at his problems, sees that the solution he seeks is not immediately before him in the discovery of the nature of enzyme action as some have thought, but must await the development of psychology into a science. We must have a method of determining the amount of psychism, a determination of the quantity in each atom, in each electron and etherion, before we can describe fully the chemistry of living things. Like the physicist, the biochemist must await the development of the final science, psychology. He cannot explain fully even the simplest chemical reaction until this side of the problem is elucidated. A means must be found to measure this hypothetical quantity of psychism possessed, it would seem, by every electron.

The beginning of every science is the measurement of the amount, or quantity of the thing it is concerned with. Hence even the science of biology is not yet born. It awaits the clear perception of the means of measuring the amount of life. Perhaps this can be measured indirectly by respiration, as Tashiro has proposed in the use of his biometer; perhaps it can be measured indirectly, electrically, by the current of action, by the blaze current as Waller suggested. But these two means remain with their importance unperceived. They are as it were the simple lenses of biochemistry. We must remember that it required centuries after simple lenses were found before men realized what these were capable of showing when combined in the form of telescopes and microscopes. How immeasurably have the telescope and the microscope extended our knowledge and conceptions of the great and the little; how immeasurably have they enriched the thought and lives of men. Consider, then, what awaits mankind in the future when once the use of the biometer has been learned, and the psychometer, that instrument which will let light into this dark world of riddle, will have been invented.

Today in the description of the universe in space and time, a description which enables us to express all physical things in terms, or dimensions, of space and time, the very dimensions of psychism are omitted, because we do not know them. Energy is expressed dimensionally as matter times the square of a velocity (ML^2T^{-2}). The dimensions of psychism should probably be added to make our description complete. But we do not yet know what its dimensions are. It must be written as (P^x). The real dimensions of energy may hence be ($ML^2T^{-2}P^x$); or with the reduction of mass to space, and of time to a fourth dimension of space (L^3P^x). Space times psychism. And since in relativity matter has the same dimensions as energy, matter also is space times psychism. In other words it is but thought. We must leave out, because of our ignorance, the psychic side of chemical reactions. Our equations, therefore, will be as incomplete as if energy were omitted. The transformation of matter and energy alone can be considered in this chapter, which becomes hence like *Hamlet* with Hamlet left out. Let us not blind ourselves to this fact.

II. FUNDAMENTAL PHENOMENA TO BE EXPLAINED

What are then these great and fundamental phenomena or facts common to all cells for which we seek an explanation hitherto eluding us? The first is that *only partially oxidized protoplasm is irritable.*

a) This is a fact long since recognized by both botanists and zoölogists alike. Deprive protoplasm of its state of partial oxidation and it loses all its fundamental properties of life. It will no longer conduct an impulse; it will not grow; its psychic life, if it have such, disappears; it will not synthesize proteins, fats, and other complex organic substances; it will no longer move spontaneously; its respiration ceases; its heat production decreases or stops. Darwin (1875) recognized this fundamental necessity of oxygen for plant irritability in *Drosera* and other plant cells. It is usually taken for granted in all physiological discussions. Seeds need oxygen to germinate; eggs need it for maturation and fertilization; embryos require it for development; the heart ceases to beat in its absence. But why is this necessary for irritability? And how is the state secured?

This state of partial oxidation is generally secured, in all aërobic forms at least, by contact of the protoplasm with the oxygen of the air; but in some forms, the anaërobic, it may be secured by contact with other oxidized substances, such as the sugars, sulphur, iron, and in some cases perhaps, as for example in the nitrogen-fixing forms, even from nitrogen gas itself. These substances are reduced by the protoplasm which thus converts itself to the state of partial oxidation necessary for its irritability and life. Forms which thus secure the state of partial oxidation from other substances than oxygen are called anaërobic forms. Probably in most cells the process of the constituting of partially oxidized or irritable protoplasm is a mixture of these two processes; in some, most of the oxidation is from the atmospheric oxygen; in others most is from other oxidized substances than oxygen. But the process, however much it may seem to differ in its details and outer aspects, is essentially the same in each. It makes no difference to the protoplasm how this state is obtained; it is the state itself which is the important thing.

The details of this process will be considered under the heading of respiration. All that is necessary here is to call attention to this fact of the state of partial oxidation as the most important thing in protoplasm, from a chemical point of view; and the most important thing for the chemist to explain. For this is the problem of the nature of respiration and irritability, that most fundamental of all vital physical and chemical properties. If we cannot understand this, we in reality understand nothing of cell life; and any hypotheses we make are sure to be no better than guesses. The problem of irritability, in other words, is not the problem of permeability, as nearly all have supposed in the past, but it is essentially a chemical problem, the problem of the maintenance of a transitory state of partial oxidation.

The first question to be discussed is then this state, for upon it all the other powers of the cell depend.

b) The second great property which we have to discuss is but one aspect of the problem of respiration just stated. It is the property cells have of synthesizing proteins, carbohydrates, fats, and other substances. In other words it is the synthetic power, the power of growth of every cell. This is a universal attribute of all forms of living matter without exception. It is the chemical process underlying reproduction. It is in virtue of this power that we have colloidal aggregates produced, which in virtue of their power of lowering surface tension and producing rigidity of surface films in the way, perhaps, pointed out by Ramsden (1919), contribute to the formation of the structures of protoplasm. Most of these syntheses, perhaps all of them, are what are known as dehydration syntheses. That is, two molecules are united or condensed together by the elimination of a molecule of water between them, a hydrogen going from one and hydroxyl from the other. In connection with this fundamental property of dehydration synthesis we must discuss also the reverse process of hydrolysis or digestion. The two processes are intimately related.

c) The third great property shown by all living cells is the property of generating electric currents. All cells have this power as long as they are alive. Every living cell is a battery. The electrical current also is part of the electrochemistry of the cell and part of the process of respiration. It depends on chemical transformations.

d) The fourth great property of all cells is their power of forming diastases or enzymes, substances which accelerate the chemical processes of cells. How do these substances act? Protoplasm is itself in the opinion of many but a collection of such diastases or enzymes. What then is a diastase? Why are they found so constantly in all cells? Why are they universal accompaniments of living matter? This again is a problem which is intimately related to, indeed bound up with, the property of irritability, growth, respiration, and the electrical phenomena of cells. All of these come down in the last analysis to the same fundamental process, namely, the process of raising the energy content of molecules and atoms. For an enhanced energy content is the real difference between the living and lifeless.

f) And finally we should consider the power of forming substances of a specific nature, since this underlies growth. I have, however, nothing to say on this subject. It is noted here for purposes of completeness and to record my ignorance.

III. THE LUMINIFEROUS ETHER, THE MEDIUM IN WHICH ALL VITAL PHENOMENA, INCLUDING THE CHEMICAL TRANSFORMATIONS, OCCUR

Before proceeding farther it will be necessary to consider for a few moments the actual position of living matter in its relation to the luminiferous ether. For the ether is the great storehouse of energy and the means by which energy is brought to the earth from the sun, to raise the energy content of the molecules of living matter to the living state.

Living things are units composed of structures; which in turn are composed of molecules or their aggregates; the molecules of atoms; and the atoms of electrons, negative and positive! Living things are, as it were, universes. Were it possible to magnify the human body so that the positive electrons would be as large as small shot, say a millimeter in diameter, that is, magnify it 10^{15} times, since the positive electrons are of the order of magnitude of 10^{-16} cm. in diameter, a man would be about 10,000 times as tall as the distance from the earth to the sun. Were the electrons luminous, each individual would look like a nebula or a collection of an immense number of suns all of which would be in rapid orbital motion. There would be constellations, which we call molecules; and the atoms would be solar systems. The electrons would be separated by wide distances, comparable to the distances between the planets in our solar system. We are in very truth minute universes composed of quadrillions of suns and planets. Who of the inhabitants living in such a universe would dream or imagine it to be conscious! The space in between the electrons would not be empty, it is believed, but filled with the substance known as the luminiferous ether. We are universes living as it were in a sea of this ether. It penetrates every part of our bodies; connects one individual universe with another.

The singular thing is, however, that there seems to be no interaction between the electrons of which we are composed, and the ether except when the electrons are accelerated. There seems to be no resistance to the passage of our electrons through this ether. When we move from place to place we do not drag the ether with us. It is a stagnant ether so far as our passage through it goes. In these respects we appear to be free and independent individuals not controlled by the ether in which we are. It is only when the relative rate of motion of electrons and ether change that an interaction occurs. That is, relative acceleration of the ether or relative acceleration of the electrons requires work.

What, then, is the ether? What are the electrons of which we are composed? What is the relation of the electrons to the ether? How do molecules and atoms get energy? How do they lose it? What is the difference between living and dead?

In what follows we may make use of ideas of the luminiferous ether advocated particularly by Sir Oliver Lodge (1909), and some of which were developed by him. There are added, however, certain definite conceptions which have been found useful in getting a clear idea of some puzzling things, although these conceptions have not yet been published and thus subjected to the criticism of the physicist. These conceptions give a clear idea of the nature of electricity, the electrons, and the relation of the ether to the electrons. I introduce them here because I am convinced that the problem of the interaction of ether and matter is not a problem for the astronomer and the physicist only, but that it is fundamental for the chemist and above all for the physiologist and the psychologist. For an understanding of the phenomena of life, something more is needed than the usual views of chemistry and physics. For life illumines physics and chemistry just as truly as physics and chemistry have illumined physiology and psychology. There is more in matter than meets the eye.

The greater universe, like the smaller, consists of space, time, and thought. The ether fills all space. It is itself four-dimensional space. It is space multiplied by time. In a paper published in the Washington Academy of Sciences (1923), I tried to show that things apparently very different had the same physical dimensions and were at the bottom the same. Thus mass, energy, moment of magnetism, all had the dimensions of space, or L^3. L is an interval in four-dimensional space. It is a length. Electric quantity, magnetic flux, and force had the dimensions of a surface, L^2. The length, L, may be expressed in centimeters, or it may be expressed in seconds. L, therefore, is an interval either of length or time. Thus all physical phenomena were expressed in dimensions of space and time.

Now the ether is space and time. It is that which fills this four-dimensional universe. It is space multiplied by time. Hence, we have expressed everything in terms of the ether. Its dimensions are then L^3T, or L^4 when we write an interval of time as L. For we live in a four-dimensional space, time being but one of the co-ordinates. The ether, then, is nothing else than energy, or mass (the two are the same) and both are space, multiplied by time. It is ergs seconds, or space seconds. It is not impossible that energy multiplied by time makes what we call thought; that their product makes psychism. Psychism may be only another name for the luminiferous ether, which is also called space and time.

Whether this space and time are limitless or limited cannot yet be said. That the velocity of light is finite and that there appears to be a finite lower limit to the length of a wave of light of about 10^{-15} cm. and to the fine-grained character of the ether may imply that the universe is not limitless. For all practical purposes, however, the ether, which we have called space and time, may be referred to as Infinity and Eternity.

All this may seem metaphysical, but it is in reality not so, for the ether is a real physical, as well perhaps as psychical, entity; and we may now consider its density, its energy content, its magnetic moment, its mass, and its velocity and quantity of electricity. Unfortunately its content of psychism is unknown. It is in fact just as real as matter itself, since it has all the attributes of matter, but in addition thereto a fourth dimension. It is this which differentiates it from matter.

The ether was postulated to explain how light is carried as well as other forms of radiant energy; how bodies may act where they are not, as in gravitation, electric and magnetic force; and to supply a need of the mind, which recoils from the barren idea that all that exists is the tenuous matter which is so sparsely distributed through space, coming from nothing, going nowhere, and doing nothing; without any means of interacting; forced by the curvature of space to move in orbits.

It is assumed that the general properties of this substance, the ether, as sketched by Sir Oliver Lodge (1909) in his book *The Ether of Space*, are known. It offers no resistance to the passage through it of bodies in uniform motion; yet it has perfect elasticity toward light and sudden impacts, and transmits radiant energy as if it were a rigid body with perfect elasticity. Lord Kelvin, Lodge, and Larmor have suggested that it is in an intense rotational movement of a very fine-grained kind, the velocity of this movement being of the order of the velocity of light in space, and that it owes its extraordinary elasticity to this fact. This is the point of departure for the following theory of its nature. I have found the following conception very useful in clarifying my ideas and they appear to me to offer a satisfactory explanation of many obscure things.

I suggest that the ether is composed of minute units which may be called etherions. It is this which constitutes the fine-grained structure of which Lodge speaks. Each etherion is in rotation about three axes, so that the surface is traveling with the velocity of light. Motion about one axis produces what is called electricity; about another, gives magnetic flux; and about the third, mechanical force. These units are 4.366×10^{-15} cm. in radius. I have supposed them spherical. The surfaces are moving at the velocity of 3×10^{10} cm. per second. The positive and negative electrons are two of these etherions which have been modified. If one of these etherions has its angular velocity increased $135/\pi$ times, it becomes a positive electron; and one which has been diminished $135/\pi$ times becomes the negative electron. In other words, the electrons are simply two etherions, one of which has lost angular velocity, that is, velocity of rotation, while the other has gained the velocity the negative has lost.

The electrons are made then by transferring a certain amount of motion from one etherion to another. This explains why there are always equal numbers of positive and negative electrons; what each is; and what their relation is to the ether from which they are formed. The radius of the negative electron is $135/\pi$ times that of an etherion, or 1.876×10^{-13} cm.; and the radius of the positive electron is $\pi/135$ that of an etherion, or 1.016×10^{-16} cm. The radius of the negative electron is the larger, for the electron is always delimited by a surface which is traveling with the velocity of light, and it is necessary to have a longer radius to obtain this when the rate of rotation is reduced. For the same reason the radius of the positive electron is correspondingly reduced below that of an etherion.

The positive and negative signs of the two electrons have to do only with the level of the *potential* of the charge in the two cases. For the amount of electricity is the same in the positive electron, the etherion, and the negative electron. In each it is 4.774×10^{-10} E.S.U. It is precisely equal in all three to the square root of $3/2$ of the centrifugal force multiplied by the radius. The potential is the quantity divided by the radius. Hence this is highest in the positive and lowest in the negative. The actual values are 4.699×10^{-2} volts in the positive; 1.093×10^{-3} volts in the etherion; and 2.545×10^{-5} volts in the negative. The electricity of the etherion is neutral. That is, it is negative to the positive and positive to the negative. The apparent repulsion of like charges is due to the fact that charged bodies at different potentials attract. Consequently each electron will attract the ether about itself. The ether thus coming between charges of the like sign moves the electrons apart, and they appear to repel each other. But the attraction between positive and negative electrons for each other will be greater than that of either electron for the ether, and these two electrons will then tend to move together, displacing the ether between them.

The ether then is composed according to the foregoing view of units each of which has a definite volume, 3.486×10^{-43} c.c.; a definite mass, namely 3.867×10^{-26} gm.; a definite energy content, that is, the kinetic energy of its rotation, of 3.48×10^{-5} ergs; a definite period of rotation, 9.144×10^{-25} seconds; and also a definite amount of neutral electricity, 4.774×10^{-10} E.S.U.

What we call its electric charge may be identified with the square root of 3/2 its centrifugal force multiplied by its radius. This product is a constant under all conditions in all the etherions and electrons. But the charge may be either positive, neutral, or negative in its potential, depending on whether the square root of 3/2 of the centrifugal force is greater than, equal to, or less than, the corresponding value of the etherion.

The density of electricity, energy, and mass in this ether thus constituted of etherions is extraordinarily great. The figures for the ether itself lie naturally between the figures of the positive and negative electrons and are in fact the geometrical mean of the two. Thus the density of electricity is enormous. There are in 1 c.c. of ether 2.868×10^{42} etherions each of which carries 4.774×10^{-10} E.S.U. This gives a density of 1.37×10^{33} E.S.U. per c.c. The density of the energy is incredibly great, namely, 9.99×10^{37} ergs per c.c. Its mass density is also very large since each etherion has a mass of 3.867×10^{-26} gm. The density of mass is 1.110×10^{17} gm. per c.c., that of water being unity. These figures are all somewhat larger than those given by Lodge in his *Ether of Space* for the reason that he has given to the ether the density and other properties of the negative electron and this is very much below that of the ether.

Each minute portion of the ether is thus moving rotationally with the velocity of light. It is not impossible that it is moving also translationally at a much slower speed and that the translational motion of the positive electron relative to the ether is 42.97 (that is, $135/\pi$) times as fast as that of every etherion, while the negative electron is moving at a correspondingly slower rate. For various reasons I have assumed also that whenever we accelerate the negative or positive electron relative to the ether, we increase its speed of rotational movement, just as the wind blowing on a windmill drives it faster as the wind's velocity increases. This increases its kinetic energy; and since energy is mass, its mass is increased. We create mass then in accelerating an electron, and mass is a function of velocity. This creation of mass, or disappearance of energy into the increase of rotational energy of the electron, is what is called inertia.

Now since the electron is only a modified etherion, it has the same dimensions as the latter, and it, too, is energy multiplied by time. Its dimensions are L^4, that is, it is space and time. Not only have the etherions a location in space, but they have a duration in time. Time means extension along a certain co-ordinate. It means existence. What a man can do depends on the amount of energy in him and the time at his disposal. He is himself space-time; he is himself ergs seconds. The very matter of which he is made, then, is energy and time. It has been set apart from the body of the Infinite, the ether.

Light I suggest is also made of etherial pulses, but these have a very short duration. The light pulses are also a kind of electrons, but larger, and they are reversible. They are but temporary, partially formed electrons. But this will be considered separately. Each light pulse contains 6.55×10^{-27} ergs seconds of ether.

Having thus obtained an idea of the nature of electricity of which we are composed and of the enormously dense and energy-containing ether in which we move, let us now seek to get a clear idea of energy.

IV. WHAT IS ENERGY? THE DIFFERENCE BETWEEN LIVING AND LIFELESS IS A DIFFERENCE IN THE ENERGY CONTENT OF THE MOLECULES

It is necessary to have a clear idea of energy, for I propose in what follows to develop the idea that the main difference between living and lifeless, between irritable and non-irritable protoplasm, is in the energy content of its molecules and atoms. That the difference between the reactive molecules of protoplasm and the same unreactive molecules outside of protoplasm is a difference in energy content. The various chemical and physical powers of protoplasm which so strikingly differentiate it from the lifeless are due to this increase in the energy content of its molecules. Living matter contains molecules having a high content of energy and capable of passing to a more stable dead form in which they contain less energy.

Anything which is able to produce this transformation from the unstable to the stable configuration is called a stimulus. A stimulus may then be defined as any process which leads to the transformation of the molecules of protoplasm from the state containing more energy, the reactive and unstable forms, to the forms containing less energy—the unreactive and stable forms. Mechanical shock, electrical currents, changes of temperature, radiant energy, and chemical substances may directly or indirectly bring this change to pass. All these things are accordingly stimuli. They cause the discharge of energy. The energy which is lost in this process is in part radiated, sometimes in the visible spectrum as in luminescent organs, and possibly in the retina, and partly in invisible radiations of longer wave-length. These softer radiations are usually absorbed by neighboring molecules and increase their motion, or heat.

On the other hand, the process of repair is the process of the restoral of this energy to its former high potential. Energy must be put into compounds to raise their store of energy to make them reactive; to convert them from the unreactive fats, amino acids, proteins, carbohydrates of ordinary nature, such as the chemist keeps in bottles, to the activated and active forms of these substances such as exist in living matter. The natures of these two forms will be considered when the various substances are considered more at length.

There are then in living matter two processes, a katakinetic which leads to the discharge of energy and the formation of stable substances; and an anakinetic, the process which restores that energy by forming other molecules with an unusual amount. By the first our molecules are dying; by the second they are endowed with life. Growth, adult life, decay! The three stages of our lives represent the shifting of the balance between these two processes.

There are then two or more kinds of every substance not alone of those in living matter. There is an inactive and an active form; an energy-poor and an energy-rich form; an unreactive and a reactive form; a stable and an unstable form.

I propose that these two or more forms of molecules or atoms which differ in their energy content be called "kinetomeres." The reactive, energy-rich ones will be *anakinetomeres;* the stable, unreactive, the *katakinetomeres*.

Since all the actions of protoplasm depend upon the content of energy of its molecules and since protoplasm is nothing else than an apparatus for transferring energy and thus, by raising the energy content of indifferent molecules, to bestow life upon them, or for prolonging the lives of these reactive forms when once created, the first thing necessary for an understanding of vital reactions is to get a clear idea of what energy is and how it is transferred and what becomes of it when it enters a molecule and transforms it from dead, stable, inactive, to the living, unstable, and highly reactive form.

What, then, is energy?

This is a concept which is not usually clearly expressed in physics. Energy is defined as that which will do work. But we are now able to make a clearer picture of energy. Energy is etherial flux or motion. The quantity of energy is the quantity of ether flowing per second.

Energy in the ether is the rotational energy, the kinetic energy of rotation, of its etherions. The electrons have energy of this same kind. They also, like the ether, have mass, and being in intense rotation have kinetic energy. They also have what is called potential energy due to the fact that being at a different level of potential from the surrounding ether they condense the ether around themselves and tend to move apart or together so that we say that they have energy of position. In reality by such movement their kinetic energy, i.e., their rate of motion, is altered. Energy is one of the phenomena of ether in motion; so also is magnetic flux and electric quantity. Energy is the product of magnetic flux by electric quantity divided by time. For while we have identified both electricity and magnetism with the square root of centrifugal force multiplied by the radius of the electron or etherion, the product of electricity and magnetism will be equal to the energy multiplied by time, that is, it is nothing else than the product of the centrifugal force by the square of the radius. Energy, hence, is nothing else than magnetic flux multiplied by electric quantity per second or it is magnetic flux multiplied by current. And it is also kinetic energy of rotation of the etherions or the electrons. It is always this under whatever guise it may appear. Energy is not only the product of electric current by magnetic flux; it may also be defined as the quantity of ether per second. It is etherial flux.

Since energy is one of the factors or attributes of the ether, the latter being nothing else than energy multiplied by time, and since we can neither create nor destroy the ether, energy can neither be created nor destroyed. Meyer, Joule, and Helmholtz proved the indestructability of energy in the physical world; Lavoisier, Rubner, and Atwater showed that the energy of living things also could neither be destroyed nor created. The law of the conservation of energy held for them as well as for the inanimate.

Energy is also the same as mass. Mass, too, is a phenomenon of rotating ether, the centrifugal force multiplied by the radius of the electrons or other etherions. When we accelerate an electron we increase its energy of rotation, that is, we increase its mass. Hence the amount of mass is a function of the velocity of translation of an electron. As already stated this increase of rotational energy, or mass, constitutes the inertia of the body. The amount of work necessary to accelerate any mass of matter is proportional to the number of electrons (positive electrons especially because so much heavier than negative) constituting that mass of matter, and to the square of its velocity. This amount of energy has disappeared at the end of the period of acceleration, and if we hunt for it we shall find it in the increased kinetic energy of rotation of the electrons of which the matter is composed. An accurate weighing of the accelerated matter would show that it has increased in weight. If we wish to find how much the mass of the material has been increased by the added energy, we have only to divide the number of ergs of energy which have passed into the body by the square of the velocity of light, since ergs are the quantity of ether flux per second expressed in electrostatic measure, and grams that same flux in electromagnetic measure. If, for example, a single erg has been used in accelerating the mass, the mass of the body would be increased by the amount of $(1/9) \times 10^{-20}$ gm. It takes, in other words, 9×10^{20} ergs to make one gram. When we say that two masses attract each other, what we are really saying is that two electrons tend to gravitate or move together proportional to the product of their kinetic energies of rotation. The mass of a positive electron is 1.662×10^{-24} gm.; this is equivalent to 1.496×10^{-3} ergs. The reason why it takes more energy to accelerate a positive electron than a negative is that more energy is required to increase the rotational energy of the positive electron, due to its greater angular momentum.

Energy, then, wherever it is, under whatever form it appears, is nothing else than the product of magnetic flux and electric current. And since magnetic flux is electric quantity times velocity, energy is always proportional to the square of electric quantity (L^4) times its acceleration (L/T^2). As energy is electricity in motion, or quantity of ether flux per second, to transfer energy we must have a means of conducting electricity, that is, of conducting the ether. It is the essence of protoplasmic chemistry that in it means have been found to conduct energy from one molecule to another. As we shall see, the substances which act catalytically, whatever their nature, do so because they are conductors of energy.

V. THE TWO FORMS OF MOLECULES AND ATOMS: REACTIVE AND INACTIVE; LIVING AND DEAD; ENERGY RICH AND ENERGY POOR; ANAKINETOMERES AND KATAKINETOMERES

Before passing on to a discussion of the method by which energy is transferred from place to place and from atom to atom, I wish now to discuss the general proposition that the molecules of all substances can exist in two or more forms which differ in their energy content and consequently in their reactions and above all in their irritability or instability and reactivity in a chemical sense. Some concrete examples of this will be given. We will make the following proposition: *All atoms, and consequently all molecules, can exist in more than one form differing in energy content.*

We will begin with the simplest atom, hydrogen. The work of Bohr permits the conclusion that this atom can exist in various states containing different amounts of energy. The energy content differs according to the size of the orbit of the negative electron which is revolving about the central positive nucleus. There are various possible orbits, and when the electron drops from an external to an orbit nearer the nucleus the atom radiates light. The different lines of the spectrum of hydrogen are due to the dropping of the electron from various exterior to more interior orbits. When the electron is in the orbit closest to the center, it has the least energy. The absorption of energy by the atom is accompanied by the electron moving from an interior to an exterior orbit. Now, of these various hydrogen atoms the most stable is of course the commonest; but the least common will be the one with the largest orbit as it is the least stable and most reactive form of hydrogen. In accordance with the suggestion already made, that form with the more energy would be the *anakinetomere;* and that with the less or the least, the *katakinetomere.* The anakinetomere form is the living form; the katakinetomere form is the dead. It is perfectly correct, therefore, from this point of view to speak of living and dead hydrogen atoms. We can even go farther with the simile if we wish and say that when the living highly reactive form of the atom passes to the dead, unreactive form, the soul of the atom escapes at the moment of its death, for a ray of light leaves the dying atom and travels onward in space, until perhaps it encounters and is absorbed by some other dead hydrogen atom, which it again raises to life by thus giving it a soul. What is this soul? It is a minute portion of the luminiferous ether; of time and space; of eternity and infinity.

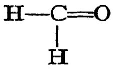
Not only hydrogen atoms are known to exist in active and inactive forms, but nitrogen, oxygen, sulphur, phosphorus, in fact all the atoms except the helium group, appear to have the same powers. Ana- and katakinetomeres of all have been more or less clearly recognized. Yellow phosphorus, with its high toxicity, is the anakinetomeric form; red is the kata form. Sulphur has several allotropic forms which differ in their energy content. Active nitrogen has recently been discovered. The ozone form of oxygen is very active.

Reference to the importance in metabolism of the activation of molecules was made by the writer in 1908, who also showed (1906) that the toxicity of all substances, salts as well as drugs of all kinds, was a function of the amount of available energy in them. Recent advances of knowledge enable us to speak with a great deal more certainty in these regards than was then possible.

It is not only the elemental form of the atoms which are thus capable of having different kinetomeres; they can also have such variations in energy content when in combination. They thus produce active and unreactive forms of compounds; active and unreactive molecules.

One of the best-known and most recently discovered instances of this kind is in the case of the sugars. The gamma sugars, we now know from the work of Irvine (1923), differ from the alpha and beta forms in their stability. The gamma sugars are so unstable that they cannot be isolated in a free state. They esterify even at ordinary temperatures and with great speed; they decompose; they pass readily to the dead, unreactive forms. Just what these gamma sugars are is uncertain; but of their instability there is no question. They oxidize spontaneously and will reduce Fehling's solution in the cold with great rapidity. Irvine has suggested that the sugar in the living plant is in the gamma form and is hence capable of the great reactivity it there shows. Certainly levulose in cane sugar is in this unstable gamma form; once split free it reverts to the relatively inactive, butylene oxide form. In other words it dies.

Another instance of the same kind is given by formaldehyde. This, as shown by Baly, Heilbron, and Barker (1921), absorbs wave-length 250 $\mu\mu$, and passes to the very reactive form, which they believe to have bivalent carbon in it. This reactive form condenses with great speed to make all sorts of plant compounds, among them the sugars and amino acids. The two forms of formaldehyde may be written as follows:



$$\underset{\displaystyle H}{\overset{}{H—\underset{|}{C}=O}}$$

Kata-formaldehyde
(Dead)

$$H—C—OH$$

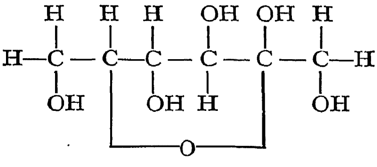
Ana-formaldehyde
(Living)

The authors suggest that even carbon dioxide exists in two forms, one of which is very reactive. Sometimes these forms differ structurally so strikingly that they can be readily distinguished, but frequently they do not so differ.

Sometimes the reactive form will only exist even temporarily under definite conditions of the medium. Thus a little alkali makes even the inert form of glucose far more reactive. Cysteine, $C_3H_7NSO_2$, is very unstable and reactive in a neutral or slightly alkaline solution, but very stable in an acid. In the alkaline solution it splits off the sulphur as sulphide, and is converted presumably into serine, the hydroxy form. In a neutral solution it takes up oxygen with great rapidity and changes to the dicysteine or cystine as it is called (see p. 59). But in very alkaline or even very slightly acid solutions, it does not oxidize spontaneously. It is obvious that as soon as the sodium salt of cysteine is formed it becomes unstable; whereas the neutral body is extraordinarily avid for oxygen; while the hydrochloride salt is very stable and the molecules are unreactive. It is obvious that the SH group, which so easily oxidizes at the neutral point, must then be in a very reactive form.

A very interesting point may be brought out in considering this oxidation of cysteine. It occurred to me that there ought to be an oxidation of any other substance which was in the solution and capable of being oxidized when cysteine oxidizes. I therefore introduced glucose into the solution hoping that it would be burned at the same time. Nothing of the sort occurred, however. The glucose remained inert. The reason for this was puzzling, but I believe it indicates that in order for any substance to be oxidized it must be in a conducting union with the oxidizer. Glucose was not in any such union. And although rapid oxidation was taking place in the cysteine beside it, it remained unaffected. Recently Hopkins (1921) has stated as the result of work in his laboratory that some fatty acids may be oxidized in these circumstances. I venture the opinion that it may be because they are able to unite with the cysteine in the amino group, or perhaps elsewhere. Cysteine, however, is very differently reactive dependent upon the degree of acidity or alkalinity of the medium. This must mean that under these different conditions cysteine molecules of quite different amounts of energy are present.

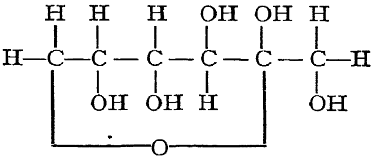
Another example of this same thing is given by levulose, $C_6H_{12}O_6$. This sugar as it is obtained in commerce has the following graphic formula:



α-*d*-Levulose

It contains, as will be observed, a butylene oxide ring. Like the other sugars it exists in two forms of this butylene ring, the alpha and the beta forms. Levulose in the form of the butylene oxide is the most stable form of levulose. It is, however, far more reactive than glucose. But it may be that this is due to the fact that there are in any solution of levulose all kinds of levuloses, the position of the ring varying from the ethylene to the propylene, the butylene, and the amylene. But the great majority of the molecules will be in the butylene form. It may be in other words that in any solution we have an equilibrium between the different forms, there being always a few molecules of higher energy content and so of greater reactivity. But in levulose there are a few more such molecules of high reactivity than in glucose and so levulose appears to be the more reactive. Be that as it may, the great majority, if not all the molecules, are in the form of the butylene oxide in ordinary levulose as Irvine has shown by his method of methylation.

The levulose in cane sugar, however, is not in the form of the butylene ring. It has been shown by Irvine (1923) that the levulose molecule here is in the form of the amylene oxide ring. This is shown in the following way. The cane sugar is methylated by means of methyl sulphate. All of the hydrogens in the hydroxyls along the chain are thus replaced by methyl except in those hydroxyls which are tied up with other molecules. When now the methylated cane sugar is hydrolyzed the tetra methyl levulose is obtained and this is the 1, 3, 4, 5 tetra methyl levulose, thus proving that the ring was between carbon atoms two and six, that is, it was the amylene ring.



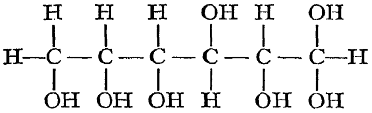
α-Amylene Oxide Levulose

As soon as amylene oxide levulose is split off from glucose, as in hydrolysis by the enzyme invertin, it passes quickly to the butylene oxide form. This is a very stable form and will not easily revert to the amylene oxide. This change from the amylene to the butylene oxide ring is accompanied by a change in the rotatory power of the solution and is part of the cause of the mutarotation which the sugar shows. There are also two forms, the alpha and the beta, of the amylene oxide ring and similarly two of the butylene. Part of the mutarotation is due to the change to the equilibrium of these two forms. Inasmuch as the usual form of levulose is the stable form and different from that in cane sugar, inversion of cane sugar by the enzyme goes to the end, and the reaction is not reversible by the enzyme.

A similar thing happens in the hydrolysis of maltose by maltase. Two molecules of glucose are produced by this hydrolysis. This transformation also goes to the end, but there is some recombination of the glucose thus set free to form another disaccharide, isomaltose.

In all of these cases the unstable form of the sugar can only exist while it is in combination. As soon as it is set free it transforms itself to the stable and unreactive form. Thus the unstable form of levulose will exist as the amylene ring levulose only while it is in cane sugar. As soon as it is free it transforms to the butylene ring form which is less reactive. In fact these unstable forms may have been produced while the levulose or glucose was in combination in the protoplasmic molecule. They are, as it were, the survivals which indicate the truth of the statements recently made that the difference between the living and the dead is this difference in reactivity. Cane sugar is to be regarded as a fragment of protoplasm in which we actually see that one of the constituents does exist in the reactive or energy-containing form.

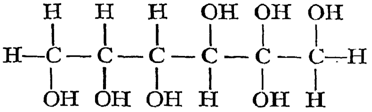
The reactive forms of the sugars are called the gamma sugars. It is suggested that they are other than the butylene oxide sugars, that is, in the gamma sugars the ring is at some other place than the butylene position. But in conversation Principal Irvine suggested that it was quite possible that the most reactive form of gamma glucose would be that in which there was no ring, but in which the terminal aldehyde group was intact. This suggestion appears on the whole very probable. The most reactive form of glucose may then be the following:



$$\begin{array}{ccccccccccccccc} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{OH} & & \mathrm{H} & & \mathrm{OH} & & \\ & & \vert & & \vert & & \vert & & \vert & & \vert & & \vert & & \\ \mathrm{H} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{H} \\ & & \vert & & \vert & & \vert & & \vert & & \vert & & \vert & & \\ & & \mathrm{OH} & & \mathrm{OH} & & \mathrm{OH} & & \mathrm{H} & & \mathrm{OH} & & \mathrm{OH} & & \end{array}$$

Possible Formula of Gamma Glucose

Similarly for levulose the reactive form will be that one in which the ketone group is free:



$$\begin{array}{ccccccccccccccc} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{OH} & & \mathrm{OH} & & \mathrm{OH} & & \\ & & \vert & & \vert & & \vert & & \vert & & \vert & & \vert & & \\ \mathrm{H} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{C} & — & \mathrm{H} \\ & & \vert & & \vert & & \vert & & \vert & & \vert & & \vert & & \\ & & \mathrm{OH} & & \mathrm{OH} & & \mathrm{OH} & & \mathrm{H} & & \mathrm{OH} & & \mathrm{H} & & \end{array}$$

Possible Formula of Most Unstable Levulose

And so on for the other sugars. Now, if this is the case, the conversion of the inactive to the active or gamma form will be produced by hydrolysis, that is, the anhydride ring will be opened, and all the hydroxyls along the chain will be free. As this state will contain more energy than the other, it is not impossible that when glucose unites with the protoplasmic molecule it receives from the latter the necessary energy and is thus able to exist for a time at least with the ring opened. The molecule if then hydrolyzed will presumably undergo all those transformations that glucose can undergo in protoplasm. Above all it will have the peculiarity of gamma glucose of greatly increased reducing powers and of powers of esterification. The gamma sugars will esterify at ordinary temperatures with great rapidity; a fundamental characteristic of vital metabolism. Furthermore they oxidize spontaneously at low temperature just as does the glucose in protoplasm. For these reasons it seems not improbable that the secret of the combustion of glucose in living cells of all kinds consists in the power of the cell to convert the glucose to its gamma or reactive form.

The evidence, however, that glucose is converted to the reactive form or the gamma form is not at present good. Hewett and Pryde (1920) report that if glucose solution is placed in contact with the living intestinal mucosa of the rabbit the solution undergoes a rapid reduction of its rotatory power without any corresponding lowering of its reducing power. The rotatory power of gamma glucose is very low. They interpreted these observations to mean that the mucosa had the power of transposing the ordinary alpha and beta mixture to that of the gamma. There is, however, a very singular thing about this if it is true. It is universally believed that gamma glucose is so unstable that it cannot be isolated. Yet here it seems to exist for an appreciable time in the solution. Furthermore if this change takes place in the solution it can only be by the action of some hydrolyzing enzyme set free from the gut. While it is quite possible that such an enzyme may exist it has not yet been found. Very recently these results have been repeated by others who report negative results. There is, therefore, a difference of results here which needs further investigation. Another similar observation has been reported by workers in Hopkins' laboratory. These observers state that the sugar in the blood during health is gamma glucose, but that in the blood of diabetics the ordinary stable alpha and beta form occurs. They report that the addition of liver extract and insulin to a glucose solution changes the glucose to the reactive form in which it can be burned. The evidence, however, is extremely unconvincing. They actually observed a slight diminution in the rotatory power of the solution of glucose on the addition of the insulin and liver extract without any change in the reducing power of the solution. It is generally believed that this evidence is not satisfactory, although it will have to be explained.

An examination of the starch molecule and cellulose and mannite fails to reveal the presence of gamma glucose. In these cases the methylation of the starch or other complex carbohydrate has yielded, when hydrolyzed afterward, only the ordinary form of methylated glucose, that is, the alpha and beta form, the butylene oxide form. Of course it might be in these cases that the original esterification or condensation was in the gamma or reactive form of the sugar which afterward was transposed into the stable butylene oxide form.

When we consider the fats and proteins we find that in no case has anyone yet isolated or shown what the reactive and unreactive forms are. But the peculiar behavior of the proteins in the cell as contrasted with their behavior outside the cell leads us to infer that something of this nature must occur. It is well known, for example, that the dead proteins appear as quite stable substances not oxidizing readily. And so also with the saturated fats. But in the cell both of these bodies metabolize with the greatest ease and rapidity. One has only to consider the intense metabolism of a man in a state of fever to realize how easily the proteins oxidize. It is known that the proteins undergo a curious rearrangement under the influence of a very little alkali by which the amino acids of which it is composed are racemized in large part. This has been interpreted by Dakin as showing that the proteins are converted to the enol form. Enol forms are usually quite active and unstable. But it hardly seems possible that the enol forms occur in the living protein. If so, we have to make some secondary assumptions to explain how it happens, if these forms there exist, that when the usual form is regenerated only one of the two possible optical isomers is regenerated. Of course it is a pecularity of living protoplasm that it contains only one of the two possible isomers of optically active substances. So that it would seem that there is something which favors the formation of only a single isomer. Perhaps whatever that principle is it may be active in the regeneration of the ordinary form from the enol form. It is not certain, then, that the enol form does not exist in the protein in the living state. But there is no evidence as yet that it does so exist. A method must be found to fix that form, whatever it is, which is there existent.

In the case of the fats, there is a possibility owing to the presence of some unsaturated fatty acids in most fats of a union with oxygen in the unsaturated fatty acid and perhaps this results in the oxidation of some of the fatty acid chains which are in the same molecule but are saturated. Nothing definite, however, can be said, and the oxidation and synthesis of the fats remains still an unsolved riddle.

Of the other substances in protoplasm which may exist in an active and an inactive form, reference should be made to those substances which are fluorescent. One of the best known of these is hematoporphyrin. This is a decomposition product of hemoglobin. Hematoporphyrin, $C_{33}H_{38}N_4O_6$, is not toxic to animals of dark skin or to albinos when the latter are in the dark. But let a white person or an albino animal receive an injection of hematoporphyrin and then be placed in the light and it shows the symptoms of an intoxication. An enormous oedema develops in human beings, accompanied by a rash which itches intensely. Similar symptoms are produced with itching in animals, and they will die unless removed from the light. Now in this case it is usual to ascribe the intoxication to the radiation of certain wave-lengths from the fluorescent substance, the hematoporphyrin. Other fluorescent substances show a similar property. Thus quinine is very much more toxic in the light than in the dark; so also is eosin, fluoresceine, and other fluorescent substances. But the toxicity is probably not due to the fluorescence as it is usually ascribed, but to the fact that by the absorption of light the hematoporphyrin has its store of energy greatly increased. It radiates always longer wave-lengths and hence less energy than it receives. It is as it were made living. This active form of hematoporphyrin, the anakinetomeric form, and similarly the active form of quinine, of eosin, and of fluoresceine is the toxic form. It is in fact so unstable that it passes very readily back to the stable form, and when it does so it radiates light of a definite wave-length, thus causing the fluorescence. But it is not probable that the light thus radiated is the cause of the toxicity, but rather the energy which it passes to any substance with which it combines.

These examples suffice to show the correctness of the proposition with which we started, namely, that molecules can exist in two or more forms which differ in their energy content, and accordingly in their reactivity.

Since the characteristic of living chemistry is its instability, we may say that protoplasm is characterized by possessing, while living, a relatively large number of molecules in a reactive, anakinetomeric form. This is the characteristic of its life. This is its life. A stimulus is anything which causes the passage of some one or several of these molecules from the ana to the kata form. At every response, then, to a stimulus, part of the protoplasm dies and becomes irresponsive. The recovery is due to the conversion of the katakinetomeric form to the anakinetomeric form. The refractory period is the period when this is happening.

We have now to ask ourselves how is this recovery brought to pass. For this is the essence of our problem, it appears to me. What is that something in our cells which raises the dead to life? Which summons Lazarus from the tomb?

VI. THE CREATION OF THE LIVING. THE TRANSFER OF ENERGY FROM MOLECULE TO MOLECULE BY RADIATION; BY ELECTRON ABSORPTION; BY ELECTRIC CONDUCTION

We are guided here by general principles. The difference is in the energy content of the molecules. Our problem is then this: How do the dead katakinetomeric molecules obtain that energy which is to make them living? We have now to go back to first principles again and ask how energy is transferred in nature.

How then can energy, i.e., electric current times magnetic flux, be transferred from one molecule to another? It may be transferred in a variety of ways.

1. It may be transferred by radiation in a series of pulses. Each radiant pulse contains a certain amount of magnetic flux and electric quantity. It contains 6.55×10^{-27} ergs seconds. If some of this magnetic flux and electric quantity is absorbed, this quantity per second is called absorbed energy. Thus the atoms of matter in the sun radiate from themselves these pulses as the solar atoms die. Some of these pulses being absorbed raise the store of energy in the molecules about us. In particular they raise the energy of the oxygen of carbon dioxide to form the energy-rich atoms of oxygen in oxygen molecules. These latter are the springs from which later we obtain all our energy. The electric and magnetic flux of the etherial pulses sent from the sun are caught by the molecules of carbon dioxide, not directly but indirectly through the agency of chlorophyll, and stored in the unstable reservoirs of the oxygen molecule. Actually the potential energy of the oxygen molecule is raised by the passing of certain electrons in the molecule to a position farther removed from the central positive nucleus of the atom, or to a position of greater instability. According to Bohr one or more of the electrons move outward so that they are

in orbits of larger radii, having a greater energy. Formaldehyde is also coincidently formed, and the carbon atom in it probably has somewhat more energy than the carbon in CO_2. The formaldehyde thus formed absorbs light energy in its turn (wave-length 250 $\mu\mu$), and is transformed into a more reactive kind which condenses at once to carbohydrates, and in the presence of nitrates makes formhydroxamic acid, amino acids, and other compounds.

The chlorophyll in the foregoing instance acts the part of a catalytic agent, that is, it acts the part of a conductor of energy from the light pulse to the molecule of carbonic acid. All catalytic agents act probably as conductors of energy. Chlorophyll is a *photo*catalytic agent, that is, it conducts the energy of light to another substance, carbonic acid. The exact nature of this process is considered a little more in detail on page 56.

2. But the energy may be transferred not by that form of electric quantity times magnetic flux known as light, as a series of pulses in the ether; it may be transferred in that form known as an electron. We have not yet discovered a method of transforming these two kinds of energy seconds, namely, an ether pulse and an electron, one into the other. Perhaps it cannot be done, although they are essentially the same in nature. But we know that molecules or atoms may part with their energy in either one of these two ways, either by radiating another pulse, that is, radiant, reversible energy-time, or by discharging an electron, a quantity of irreversible energy-time. For this electron, or permanent ether pulse, to be shot out from the atom a considerable quantity of energy-time must usually be absorbed. The electron usually leaves at a rather high, indeed at times at a very high, velocity. The velocity with which the electron is radiated depends upon the amount of energy absorbed, that is, it depends upon the energy content of the ether pulse which is absorbed. For since, in all ether pulses the total product of energy time is a constant, some pulses will have a great energy content and little time; others will have a small energy content and a large amount of time; that is, in the one case the energy content is high but the period of duration is short; whereas in the other the energy content is low but the period is long. The velocity of expulsion of an electron is then dependent upon the energy content of the pulse which expels it. So the very short waves of great energy content of the period corresponding to X-rays cause the expulsion of electrons with a very high velocity; while the feeble rays of the red spectrum expel them at a very low velocity. All of this is of importance in understanding color vision. It is well known that the shorter waves, with the greater energy content, those in the violet and ultra-violet, produce very intense chemical changes. Thus, for example, the energy of the silver atom is so greatly reduced by exposure to ultra-violet light that it is no longer toxic or poisonous. It becomes the inert metallic silver. By the action of light an electron is transferred from another atom to the silver atom leading to a far more stable arrangement of the electrons of the silver. The radiations of electrons under the influence of light are beta radiations. They come from

certain atoms under certain conditions. Negative electrons may be given off from almost every form of matter. And their absorption may either raise or indirectly lower the store of energy in that atom or molecule which has absorbed.

Thus, according to Zwaardemaker (1921*b*), potassium owes its peculiar action in protoplasm to the fact that under the influence of light it is photoelectrically active and gives off negative electrons. Zwaardemaker states that it is possible to replace potassium in its physiological rôle in the frog's heart by an amount of any other element such as uranium, which has an equal discharge of electrons.

The action of X-rays is generally attributed to their powers of producing a radiation of electrons at a high velocity from certain atoms of the tissues through which the X-ray passes. An X-ray thus has a double effect, namely, a direct effect upon the atom which has lost the electron and also an indirect effect due to the electrons which have been shot out of one atom changing the state of another which picks it up. As the electron is not radiated in the direct line of the X-ray, a scattering of electrons is thus produced which increases the radius of the area affected by the X-ray. Inasmuch as the velocity of the expelled rays depends upon the energy content of the X-rays absorbed, and this is higher the shorter the wave-length, or, as is generally stated, the harder the ray, so the shorter, harder more penetrating X-rays produce an electronic discharge at a higher velocity than do the softer. The secondary radiations thus produced as they have a higher velocity will radiate farther before absorption. So the harder the ray, the greater will be the cross-sectional area as well as the depth of the tissue affected by the ray.

Inasmuch as the more reactive tissues have their molecules in the least stable form, these tissues will be the easiest to effect by X-rays. Thus, dividing cells in which the metabolism is very intense, as Lyon showed, are very easily destroyed and cancer tissue also. But there will be a boundary area in any X-ray irradiation in which the anakinetomeric molecules will be increased. If this boundary is cancerous the ray here may increase cancerous growth.

3. But the third, more common, and easier way to transfer energy is by means of conductors of electricity. To set up a pulse in the ether requires either a great deal of energy acting for a short time, or a small amount acting for a long time. The value of h, the minimum amount of energy times time which must be accumulated before radiation can occur, is 6.55×10^{-27} ergs seconds. Similarly to discharge electrons by radiation requires a considerable increase in energy, in many cases at least. Between substances, however, at a very slight difference of level, or potential, of energy an exchange may take place if only they are connected by what is called a conductor. If two charged plates of a condenser at a different potential are connected by a metal, the electric potential is equalized. The metal acts the part of a path interposing little resistance to the flow of electrons along it. For such an exchange to occur, energy must be at a difference of potential.

Now imagine the condenser plates reduced to the size of two molecules each of which contains free energy but at a different potential. If only we could connect these two molecules by a conductor, energy would pass from one to the other. Such conductors exist. We call them catalytic substances. Or the energy may pass directly, the substances uniting in a conducting union with each other. In either case, *for such an interchange of energy between molecules to occur the union must be of a conducting nature.* The two molecules must make such a union as to permit electrical charges to flow from one substance where they are at a higher potential to another molecule or atom where they are at a lower. In all ordinary exchanges of energy in chemical reactions of every kind, it is necessary hence for the two molecules or atoms so to unite as to make good conductors of electricity. If the molecules cannot themselves so unite it is necessary that each unite with a third, which thus is interposed between them as a conducting system. In all such cases as these the potential energy of the system is due to an electron placed in a certain position. By transferring that electron to another system the one system loses potential energy, the other gains it. It is because electrons have to be transferred in this method of carrying energy that the transferrer must be a conductor of electricity.

VII. WHAT IS A MOLECULE?

We come hence to the next question, namely, what must be the nature of the union between molecules in order that there shall be a transfer of electrons? Are all unions of this nature? Physical as well as chemical? Or is it only in chemical unions that the potential energy of one molecule, that is, of one system of atoms or electrons can pass to another? This brings us to the necessity of clearly defining a molecule and getting a clear conception of chemical affinity and chemical union. The particular problem we are trying to solve is this: How can the energy get from the oxygen molecule, where it has been put by the sun, to one or more atoms of the substances in protoplasm, so as to vitalize them?

We must now get a clear conception of what a molecule is and what is the nature of chemical affinity and of chemical combination. For there is a fundamental and most important difference in kind and character between chemical molecules and aggregates of such molecules to form micellae or genes or other hypothetical protoplasmic units. This difference is usually not clearly perceived, and many have made the mistake of considering chemical unions and unions due to cohesion, for example, as if they were of the same kind. Clear ideas must be had on these points before any clear picture of what is happening in protoplasm can be arrived at. It is because ideas have not been clear on the nature of a molecule that the confusion prevailing in the field of adsorption is due. There is a clear-cut difference between molecules and physical associations of molecules.

It is now generally believed by chemists that the union of atoms to make molecules is by the atoms sharing electrons between them. This conclusion may be accepted as extremely probable if not entirely proved. Thus, in oxygen the two atoms have in common certainly two electrons. It cannot yet be said whether these two electrons are revolving about both atoms, as is quite possible and to the writer probable, or whether the two electrons are stationary or approximately so and lying between the atoms. Each so-called bond in chemistry, represented by the chemist as a dash, really consists of two electrons (Thompson, Parson, Lewis, Langmuir). *The essential thing is that these shared electrons belong to the electronic systems of two atoms at the same time. Thus the two atoms intrapenetrate each other.* This is the vital point. A molecule of oxygen is essentially a great atom of oxygen, only it differs from the ordinary oxygen atom in that it has two centers of organization in place of one. We may perhaps find that the inner electrons of each atom are revolving only about their own atomic center; while the outer or so-called valence electrons are revolving about both. This is as yet uncertain, but is referred to later.

We may define a molecule, therefore, as an intrapenetrating system of atoms.

This kind of a system is fundamentally different from a system composed of molecules. Let us examine in what the difference consists. A system of molecules (such as a crystal) is formed by the forces of cohesion. The two or more molecules cohere; *they lie side by side, but their atoms do not penetrate from one molecule to the other. The two molecules share no electrons in common* as they do in chemical unions. Adhesion or cohesion is then something quite different from the force of chemical attraction.

Since atoms in molecules are intrapenetrating, in that they share electrons in common, the condition within a molecule is essentially the same as within an atom. A molecule is essentially a polycentric atom; and an atom is a monocentric molecule. In an atom there is but one center of organization—one positive center—while in a molecule there are two or more such centers. Our solar system is essentially an atom, although the planets have electron moons which circulate only around them. But were there another sun and if Mercury, Venus, and the earth circulated around our sun, while Jupiter and the outer planets circulated about our sun, and another sun at a distance, as perhaps the comets do, then there would be an analogy with the foregoing picture of a molecule.

Now, if in any molecule we start within an atom, we can traverse the whole molecule without passing outside of an atom at any point. We should remain within the atomic fields everywhere. This is, I believe, a very fundamental point, for the conditions within atoms appear to be different from the conditions without atoms. It is essentially different from the conditions in a micella or gene. There in passing from one molecule to another, one must pass outside the atoms.

If, then, the atoms are conductors of electricity and have no resistance, then the molecule is also a conductor and has no resistance, since in passing through it one never leaves the atoms. One has entered always a second atom before leaving the first.

If, however, one passes from one molecule to another, even though these molecules are side by side, held by cohesional forces, one must pass outside the atom to go from one to another, for such systems are not mutually penetrating.

Thus there is a fundamental difference between aggregates of atoms to make molecules and aggregates of molecules to make crystals. The force of cohesion differs from that of chemical affinity.

What holds atoms together to make a molecule is not, properly speaking, either their electrical or magnetic attractions for each other. It is rather owing to the fact that by sharing between them some of the electrons of each, the two atoms thus united contain between them less energy than when they were separate. The force of chemical attraction is hence the tendency to form more stable electron configurations in the atoms. It seems as if the octet was a peculiarly stable arrangement. The reason, according to Parson (1915), is that the electrons have magnetic attractions for each other. But in any case this arrangement seems in virtue of the electrical forces between the negative electrons and between the electrons and the positive center, and their magnetic forces, to be most stable. So that both magnetic and electrical forces come into play. *The attraction is not, however, from atom center to atom;* but a regrouping into a more stable form of the electron groups in each atom. Always it seems that atoms or systems of electrons which, could they fuse, would reduce their potential energy, and which have themselves energy at a different potential, tend to move together into places and positions toward each other in which their potential energy is at a minimum.

On the other hand, the force of attraction between molecules is not due to this possibility of sharing electrons and thereby forming more stable configurations. It is not chemical affinity as has been supposed, but it is a force more analogous to that of gravitation, which indeed it involves; or to magnetic moment. Quantity of cohesion does not have the dimensions of L^2, the dimensions of electric quantity and of magnetic quantity, but it has the dimensions of L^3, that is, it has the dimensions of mass or of moment of magnetism. The quantity of cohesion in a molecule is proportional to the product of two things, namely, the number of electrons held in common between two or more atoms, and the gravitational mass.

From the cohesion of a molecule we can indeed compute how many of the electrons are shared by two atoms. From this it appears that these electrons when thus held in common between atoms are in some sort of a different state from the other electrons. From the fact that it is these electrons, as shown by Drude (1904), which are the cause of the action of the molecule in dispersing visible light, we may infer that the orbits of electrons thus held in common are wider than are the orbits of the other atomic electrons which are nearer the centers of the atoms. This leads to the conclusion, suggested on page 39, that these electrons are circulating about two or more atomic centers rather than one. Having thus orbits of larger radii, it is these electrons which will be synchronous with light rays. They will also have the greatest moments of magnetism of any of the electrons in the molecule. The valence electrons must hence determine the magnetic or diamagnetic properties. This agrees with the work of Pascal (1911) who showed that the diamagnetic properties of molecules depended upon the number of valences, that is, according to the foregoing, upon the number of shared electrons.

Arguing from this reasoning that cohesion should be due to the combined action of gravitation and magnetic moment, the author found, indeed, that the cohesion of a single electron, that, namely, of the positive electron or proton, could be calculated from the square root of the product of the moment of magnetism by the mass of the proton. In fact this quantity of cohesion which was $\sqrt[2]{3.22\times10^{-37}}$ cohesional units, the quantity of cohesion in a proton or positive electron, was precisely equal to $\sqrt[2]{27\pi^2\ c\times\text{moment of magnetism}\times\text{mass}}$. The mass of a proton is 1.662×10^{-24} gm. and the moment of magnetism according to my calculations is 2.426×10^{-26} magnetic units cm. The combined effect of the gravitational mass by the moment of magnetism is what is known as cohesional quantity. It is obviously something quite different from chemical affinity, although in certain particulars it resembles it.

Concerning any structure in protoplasm we have to inquire, then, whether it is a molecule, or whether it is a structure built up by cohesion. The properties of these two things will be quite different in that in the one case the conditions are essentially those of intra-atomic existence. In the other case we have to pass outside of the atoms as we pass from molecule to molecule.

THE ELECTRIC CONDUCTIVITY OF MOLECULES AND ATOMS

Since our problem in living matter is the conduction of energy from oxygen to the atoms and molecules of which living matter is composed, the electrical conductivity of molecules becomes of great importance. The work of Kammerlingh Onnes on the conductivity of metals at very low temperatures proved that resistance falls almost or entirely to zero at or very near absolute zero. This fact proves that the atoms of metals, at any rate, are perfect conductors of electricity. For as the temperature rises and the atoms separate, resistance increases. From this it is inferred that resistance is met with in passing from one atom to another, but not within the atom. If, however, the atoms are brought so close together that they intrapenetrate, their resistance disappears.

Of other atoms than metals there is no doubt that the atom of carbon is also a good conductor. This is shown by the fact that graphite is a good conductor of the first class. It conducts as if it were a metal. And, indeed, since every electron moving within an atom is an electric current, and we know that there is no resistance to the constant flow of such currents in an atom, since if there were the electron would ultimately fall to the center and come to rest and the atom as such would perish, we see that every atom must be a perfect conductor of electricity. Within the atom, therefore, there is no resistance. But on leaving it there may be resistance. In free space, however, there is no resistance. To start an electron in motion, or to increase its motion, requires that work be done. The reason being that the mass of an electron is increased when it moves. The energy expended in giving it motion reappears as its increased mass. But once moving at a uniform rate there is no frictional resistance to be overcome. An electron thus moving at a constant velocity requires no work to keep it moving. There is no resistance to its passage. So in a metal the atoms of which the wire is composed are supposed to have associated with them a certain number of very loosely bound electrons. At very low temperatures as in Onnes' experiments, if all these loose electrons are started by means of a magnetic field in movement in one direction, the wire being a ring, the atoms are so close together that the loose electrons pass freely from system to system without resistance and the current will continue to flow indefinitely.

While it is certain that the atoms of metals are practically perfect conductors gaining and losing electrons with no loss of energy, it is difficult to decide whether this is true of all atoms or not. Certainly it is true of carbon atoms. In oxygen and the non-metallic elements the atoms form molecules of two atoms each. There is certainly a resistance in the passage from molecule to molecule, since even liquid or solid hydrogen or oxygen are not good conductors. Metals are substances of which the atoms gain or lose electrons with little change in energy.

What will be the condition as regards conductivity within molecules? Certainly if the atoms of which these are composed are conductors and if the molecule is made of intrapenetrating systems of atoms, then the molecule also must be a conductor of the first class. Probably no one would doubt this for a molecule of a metal; and it is equally certain for all molecules composed of carbon atoms. A stick of highly compressed graphite is a good conductor, and the chains of carbon atoms are nothing else than very minute and highly compressed rods of graphite. We may then conclude that certainly molecules made of carbon atoms must be good conductors. It is not, however, sure that when an oxygen atom is intercalated between two carbons, as for example in the esters or disaccharides, or a nitrogen atom between carbons, as in the proteins, that the current can pass as freely through the oxygen or nitrogen as it can through the carbon chains themselves.

The fact that protoplasm is composed so largely of molecules consisting of chains of carbon atoms which are hence conductors of the first class is a matter of great importance in understanding the electrical phenomena, catalysis, and synthesis of cells.

To resume then: Oxygen is the great reservoir of energy for all living things except plants which are able to get their energy from the sun. The oxygen atoms of carbon dioxide received this energy from the sun when the carbon dioxide was in union with chlorophyll. The carbon dioxide was in fact a reservoir into which a small part of the energy of sunlight was poured and is held. The energy is in the oxygen atom in the form of certain configurations of the electrons. If an oxygen atom can acquire two more negative electrons it passes into a more stable, unreactive form, in which it is electronegative. To obtain, then, the energy from oxygen it is necessary to conduct into it two negative electrons. The process of oxidation consists in thus taking negative electrons from the oxidized substance and transferring them to the oxygen.

By this transfer of energy from the oxygen atom two things may happen. Part of the energy may be given off as heat and dissipated as kinetic molecular motion. But part *of the energy may be retained in the receiving molecule which is thus rendered more reactive.* It is this latter condition with which we are concerned in protoplasm. A part of the energy is retained to be later dissipated under the influence of what is called a stimulus.

IX. THE NATURE OF IRRITABLE PROTOPLASM. ORIGIN OF ALL LIVING ENERGY IN SUNLIGHT AND OXYGEN

In the light of the preceding we may now make the following picture of the process by which the irritability of protoplasm is maintained and its peculiar chemical powers and processes are created. Oxygen with its high store of energy comes to the cell membrane, to the cell protoplasm. It unites in a molecular union with this protoplasm, that is, it unites about as it does in the union with hemoglobin. The only difference is that the union is not so easily reversible. The unions are not dissociated to the same degree. Protoplasm itself, or at any rate its limiting membranes wherever they occur, consist of molecules of carbon chains, which thus constitute conductors of the first class. It is probable that the oxygen union takes place with some unsaturated bonds in these carbon-chain molecules. These molecules are often, if not always, oriented in the membrane so that the membrane has a definite structure. The food substances, that is, the substances which are to receive the energy of the oxygen and thus to be made reactive anakinetomeres, unite with these same molecules of the membrane but on the inside. We thus have a conducting system formed, consisting of the oxygen molecules containing energy, the protoplasm or rather certain constituents of its molecules, and the empty energy reservoirs, i.e., the inactive katakinetomeres: amino acids, glucose, fat, etc. This union being a conducting union, there then occurs a partial flow of energy in the form of an electric current between the oxygen and the receptive molecules so that the latter are raised to the reactive anakinetomeric form. *It is this unstable combination of partially oxidized protoplasm which is the irritable substance in protoplasm.*

This flow of energy has been in the form of a flow of electricity inward from the oxygen molecules, although only a portion of the energy has actually passed. This is the cause of the electrical phenomena of protoplasm and the reason for the constant difference of potential between the interior and the exterior of the cell which persists as long as the protoplasm is being oxidized.

Any stimulus causes the consummation of the oxidation. That is, it causes the flow of the remainder of the energy from the oxygen to the protoplasm. This flow being nothing else than an electric current passing inward is the current of action. This is discussed more in detail on page 66.

All the chemical phenomena of the cell are due to the reactions of the activated substances which have received the energy directly or indirectly from the oxygen atom and whose electrons, or some of them, have in consequence moved outward to new and less stable orbits. In other words, it is the oxygen which thus indirectly carries the energy from the sunlight to the atoms or molecules which it thus activates. It is as it were the breath of life which the oxygen breathes into the atoms of the cell. Sunlight thus creates life today just as it always has done in the past.

The whole matter may be summed up in the statement of a general nature leaving the details to be worked out later, that in protoplasm or living matter we have a self-perpetuating system for the transfer of energy from oxygen, or other sources, to inactive molecules which are thus made unstable and chemically active.

This completes our examination of the first great peculiarity of protoplasm, namely, that only partially oxidized protoplasm is irritable. We see why this is so. It is that oxygen brings to protoplasm the energy necessary to raise its dead inert inactive molecules to the anakinetic, living, irritable, chemically reactive forms, thus making irritable protoplasm and providing for metabolism, growth, and reproduction.

We will now examine more in detail the process by which oxygen combines with protoplasm, or the molcules of the membranes, in other words, the process of respiration, and the production of carbon dioxide.

X. RESPIRATION

By respiration is meant usually the gaseous metabolism of protoplasm; that is, its consumption of oxygen and liberation of carbon dioxide. But in a broader sense the word may be used to designate all the processes of oxidation in protoplasm whether these are produced by atmospheric oxygen or other substances. The botanists also use the word as covering all the production of carbon dioxide whether this is by the process of alcoholic fermentation or not. We shall here consider these two processes separately, since they are not necessarily synchronous as to time. The liberation of carbon dioxide may take place long after the oxidation has occurred. The oxidation is, however, the important part of respiration; the liberation of carbon dioxide is a secondary result of it.

The facts are known to all. Living matter constantly consumes oxygen. Fats, sugars, and proteins brought into living matter are rapidly and readily burned there at low temperatures. In the case of some animals of the arctic waters this combustion occurs with rapidity enough to support their life at a temperature but little different from zero. The question which we have to discuss is how this becomes possible? How is it that these stable, unreactive substances burn so readily in living matter, but not outside it?

One great feature of this problem has already been discussed, namely, the methods by which the substances in protoplasm are activated. The broad principle was laid down, that the oxidation was produced by the fact that protoplasm was capable of uniting into a conducting system oxygen (possibly in the form of peroxide) with its store of energy, on the one hand, and the substances to be burned, on the other. By this union the substance to be burned is changed to a reactive form by which it either, when stimulated, receives more energy from the oxygen, or becomes capable of directly combining with oxygen itself. There seems no doubt that in a general way this represents the facts; but we have now to inquire into the progress which has been made in the detailed examination of the process.

There are evidently two processes to be considered. One is the increase in the oxidizing power of the oxygen which in the molecular form is rather stable; the other is the increase in the reducing power of the substances to be burned. By power is here meant velocity of oxidation or reduction. The problem of the increase in reducing power of the substances to be burned has already been treated. It consists in the activation in some way or other of the reducing substances—their conversion to the active form. Oxygen also may be put in a more reactive form since it naturally exists in the molecule in the most stable form.

1. *The rôle of water in respiration:*

Let us now in the first place turn our attention to oxygen and its relation to water. We must know what happens between oxygen and water, since the oxygen is dissolved in water. Water is for some reason necessary for oxidations by molecular oxygen. The oxygen is as it were activated by the water, or changed to some compound which will combine with protoplasm or some of its constituents. The fact that water is necessary for oxidation is known to all. Iron rusts rapidly in moist air, but not at all in dry. H. B. Baker (1902) showed that perfectly dry oxygen and hydrogen would not unite even at a temperature of 1000° C. The fact that oxygen is decidedly more soluble in water than an inert gas such as helium shows that it must enter into some sort of union with the water. There are several compounds of oxygen with water known. One of these is hydrogen peroxide, H_2O_2, and another is ozonic acid, H_2O_4. The former of these compounds is certainly produced in the course of many oxidations, as Traube and many others have shown. The latter, ozonic acid, was obtained by the action of ozone on a 40 per cent solution of potassium hydroxide in a freezing mixture by Baeyer and Villiger (1902). So far as I know it has not been shown that it exists when oxygen dissolves in water. Hydrogen peroxide probably does exist under those circumstances, since traces of it are found in the atmosphere. It is formed indeed by the action of light on moist air.

In this field where so much is dark we must be guided by analogy with other substances. One such substance which resembles oxygen is bromine. When bromine enters water it unites with it. This is shown by the great solubility of bromine in water. There is formed also hypobromous acid, HOBr; hydrobromic acid, HBr; and ultimately bromic acid, $HBrO_3$. Since no transfer of charges or reaction can occur between the water and bromine until a chemical union has occurred between them, the first step must be a union of Br_2 with water. Unfortunately we do not know whether this union is between H_2O, or the molecule H_4O_2, or the trihydrole, H_6O_3. If it is the first or the second we would have the equations:

$$Br_2+H_2O \rightarrow HBr+HBrO.$$

If, on the other hand, it were with $3H_2O$ we would have the result:

$$(1) \qquad 2Br_2+(H_2O)_3 \rightarrow Br_4(H_2O)_3$$

$$(2) \qquad Br_4(H_2O)_3 \rightarrow 3HBr+HOBr+H_2O_2.$$

In the latter case then we would have a certain amount of hydrogen peroxide formed; in the first case not. Now if we examine HBr, HOBr, and H_2O_2 to see which of these has oxidizing powers and just where this power is in the molecule we find the following facts. Hydrobromic acid, HBr, has as is well known relatively weak oxidizing properties. It has no more powers of oxidation, or but little more, than has hydrochloric acid. Nevertheless, it has some powers. It will oxidize zinc, for example, or any metal above lead in the scale of solution tensions. It will oxidize magnesium, cadmium, cobalt, sodium, potassium, for example. The oxidizing agent in it is the H ion. This ion has a positive charge of electricity which it will part with to some other substance, such as zinc, oxidizing the latter and being itself reduced to atomic hydrogen, that is, to neutral hydrogen. It has the property of taking up a negative electron in other words and by so doing it oxidizes that atom which loses the electron. The potential of its oxidizing power being that of a H ion, it is exactly the same as that of water itself since the latter also contains H ions. But the rate of oxidation will be much greater in the acid since the number of these ions will be greater in the acid. It should be noticed, since it is so often overlooked, that the oxidizing powers of water are not due to the oxygen of water but to the H ions it contains. So much, then, for the hydrobromic acid. This is not the oxidizing agent we seek since the potential of its positive charge is too low to accomplish the oxidations which bromine dissolved in water will accomplish. In this connection it may be pointed out that the actions of all ions are largely a function of the potential of their electric charges, that is, of the ionic potential (Mathews, 1904).

Of the other two substances hydrogen peroxide certainly has oxidizing powers, but they are on the whole below those of bromine. It acts, as is well known, as a reducing agent. It reduces potassium permanganate, silver nitrate, and other substances, among them hypobromous acid. Its oxidizing potential is about that of cupric compounds. It will, however, rapidly oxidize many organic acids, such as formic, lactic, tartaric, malic, and others in the presence of a ferrous salt; and it burns sugars, or some of them with great rapidity in the presence of a ferric salt (Fischer and Busch, 1891). According to Kastle and Loevenhart (1903), it unites with the substances undergoing oxidation, and subsequently decomposes. Certainly in the presence of iron salts hydrogen peroxide is a strong oxidizing agent. Part of the oxidation may be due to this substance, then, if it is formed when bromine dissolves in water, but the fact that it so rapidly reduces hypobromous acid is evidence that it is formed only in small amounts if at all.

The third substance is hypobromous acid, HBrO. There is no question that this acid in the free form is an intense oxidizing agent, and it alone would suffice to explain the oxidizing powers of bromine in water. Sodium hypobromite has strong powers of oxidation, but these are enhanced when the solution is made neutral or very faintly acid. Now, since hypobromous acid is a very weak acid, it forms few H ions and BrO ions. Neither of these have a sufficient oxidizing potential to explain its action. There is now little doubt that, like water, it dissociates both as a base and as an acid. It forms also bromine hydrate, BrOH, which presumably dissociates into positive bromine ions, Br^+, and negative hydroxyl ions, $\overline{OH}$. The oxidizing powers are due to the bromine which has a positive charge at a very high potential and is hence an intense oxidizing agent.

If, now, we turn to oxygen and write similar equations for the behavior of oxygen when dissolved in water, we would have:

$$2O_2 + 2H_2O \rightarrow H_4O_6 \rightarrow (OOH)_2 + H_2O_2.$$

Here, again, we have hydrogen peroxide, which might be the oxidizing agent. But ozonic acid, H_2O_4, would be a very much more intense oxidizing agent if it exists in water in minute amounts. It is substantially oxygen hydroxide, with the one oxygen electropositive. This substance would act just like the bromine in bromine hydrate. There is, however, so far as I know, no evidence that it is formed under these conditions, although it would seem from general principles almost certain that some must be present. If present, it would act in all particulars like cupric hydrate for example, only it would be a far more intense oxidizing agent as the oxygen has a greater tendency to lose its positive charge, that is, it has a higher ionic potential than has the cupric ion.

We have a choice then between the two possibilities either:

(1) $$O_2 + 2H_2O_2 \rightarrow 2H_2O_2;$$

or

(2) $$2O_2 + 2H_2O \rightarrow H_4O_6 \rightarrow H_2O_4 + H_2O_2.$$

Possibly both of these reactions occur when oxygen dissolves in water. In any case the amounts of ozonic acid, that is, of oxygen hydrate, and of hydrogen peroxide are small. And presumably there is an intermediate stage of a molecular union, H_4O_6. But no definite statement can yet be made on this point which is so important for an understanding of the process of respiration. Suffice it to say that something of the nature of that just described must occur, if our present conceptions of chemistry are at all correct. At any rate it makes clear why water is a necessity for the oxidation. The point of equilibrium must be far over to the left-hand side, that is, equilibrium must be had when there is very little ozonic acid and hydrogen peroxide in the solution.

Another interesting fact may be brought out, namely, the effect of a change of hydrogen and hydroxyl ions on the rate of the oxidation if that is due to the ozonic acid functioning as a base. The concentration of the positive oxygen of ozonic acid will be at its maximum close to the neutral point. For the addition of alkali will greatly reduce the ionization as oxygen is so weak a base; and, on the other hand, the addition of hydrogen ions, that is, of acid, will greatly diminish the number of hydroxyls as the product of the hydrogen and hydroxyl is a constant. Such a reaction would be at its greatest efficiency at just about neutrality, that is, at the reaction of living matter.

Since the oxygen hydrate, if it exists, will be a very intense oxidizing agent and since at any instant there is but very little of it present, the rate of any oxidation it can carry on will be limited by the speed with which the ozonic acid, or the hydrogen peroxide can be formed from the dissolved oxygen. Nothing is known to the author about the speed of this reaction. There is, however, in all cells a catalytic agent which certainly accelerates the reaction in the left-hand direction if it be added to hydrogen peroxide, and which also, if this reaction is reversible, must hasten the speed in the other direction also. This catalytic substance is the diastase, *catalase*, which is universally found in all living matter. It would seem probable that the function of this somewhat enigmatic substance must be to accelerate the formation of hydrogen peroxide and ozonic acid from water and dissolved oxygen. But before taking up this phase of the matter, I wish to consider certain other catalytic agents which so powerfully accelerate oxidations by peroxide solutions.

2. *Iron and manganese and their rôle in respiration:*

Iron is found in all cells, both of plants and animals. Its function is unknown except that it is necessary for the formation of chlorophyll and hemoglobin. It is not a constituent of the molecule of chlorophyll, but in its absence chlorophyll is not formed, plants with deficient iron being pale. On the other hand, iron is part of the hemoglobin molecule. In its absence hemoglobin cannot be formed and the disease known as chlorosis is the result. But iron is found in all cells whether they produce hemoglobin or not. It occurs there, as MacCallum has shown, partly in the free and partly in the masked form, that is, partly in unions which set free the iron ion and partly in organic combination. Recently by MacCallum's reaction for iron with potassium ferrocyanide, the Prussian blue reaction, its presence has been shown in the nuclei and Nissel substance of nerve cells.

Ferric or ferrous iron accelerates many oxidations due to hydrogen peroxide. Mention of these has been made above. The oxidation of organic acids, such as lactic for example, by peroxide is accelerated by ferrous iron; and of the sugars also. How then does it act? These cases are extraordinarily interesting, for they are the substances found in cells and which are oxidized there; and as Dakin has shown, the products of peroxide oxidation are just those found in living things; that is, peroxide and living protoplasm will produce about the same oxidation products. Living protoplasm and peroxide, in other words, have toward many substances the same oxidation potential. Hydrogen peroxide reduces ferric salts to the ferrous state. This is shown by the fact that when added to a solution of ferric chloride and ferricyanide it produces a blue precipitate of Prussian blue. This means that a ferric atom has a positive charge at a higher potential than does hydrogen peroxide. It takes a negative electron from the oxygen atom of hydrogen peroxide, raising the energy content of the latter. Now, since ferrous iron catalyzes the oxidation of organic acids by hydrogen peroxide and since a charge probably cannot be transferred unless the compounds are in union, it is probable that this catalysis is due to the fact that the iron atom unites the peroxide on the one hand with the acid on the other. But this will not explain the matter. Certainly the oxidizing potential of ferric iron is not sufficient to oxidize lactic acid. This is shown by the fact that ferric lactate is stable. Now the oxidizing potential of hydrogen peroxide is lower than that of ferric chloride, as is shown by the fact that ferric chloride is reduced by the hydrogen peroxide. The catalytic action cannot be thus explained. There is, however, another possibility. Ferric salts decompose hydrogen peroxide, thus setting free molecular oxygen. They thus will destroy the equilibrium of the reaction by which peroxide is formed and this will lead to the formation of more oxygen hydrate. They thus in reality may catalyze the reaction by which ozonic acid is formed. It is the latter which unites with the lactic or other acids directly or through iron and carries out the oxidation. This reaction again needs study before its mechanism will be more than conjecture. The catalytic action of ferrous or ferric salts toward the oxidation of glucose and other sugars would be similarly explained. At any rate the important fact is that iron catalyzes reactions in which hydrogen peroxide or ozonic acid take part, in the system oxygen-water, and its action in the cell is no doubt connected with this property. It probably acts in this way in the hemoglobin molecule, as is shown by the fact that hemoglobin or hematin or iron salt itself will catalyze the oxidation of guaiac, guaiaconic acid, or benzidene in the presence of hydrogen peroxide.

Manganese dioxide in traces will decompose hydrogen peroxide. This compound in the presence of certain organic acids forms the diastase known as "laccase," according to Bertrand. Laccase oxidizes the coloring matter of the lac insect. This coloring matter is at first red, but by further oxidation turns black, much as tyrosine is oxidized by tyrosinase. The exact method by which this oxidation is produced is unknown.

The noble metals, such as platinum, also catalyze hydrogen peroxide and lead to the oxidation of substances which are in solution. The mechanism of this action is uncertain.

In all these oxidations there is a singular fact. Glucose, for example, will only oxidize spontaneously if it is in an alkaline solution. Then, if shaken with air or if air is blown though its solution, it oxidizes very rapidly. Now, there is no lack of oxidizing power in the system of oxygen and water. This is shown, for example, by the rapid spontaneous oxidation of cysteine under such circumstances. Why, then, does the glucose not oxidize in water? It can only be that it is in too stable a condition. But if we add a ferrous salt to the peroxide solution it oxidizes very fast; so fast that it will heat the solution. Is there some action, then, of the iron salt on the glucose? Is it possible that it is changed into the reactive form? Were this the case it should oxidize spontaneously in the air if iron is added to it, but this it does not do. We are evidently at an impasse and further work is necessary.

Among the other oxidations which are catalyzed by iron and which are occurring in cells, special mention may be made of the oxidation of cysteine. This is a spontaneous oxidation carried out at a rapid rate by the oxygen of the air at the neutral point under conditions identical, as shown by the author, with those in living cells. This reaction is remarkably catalyzed by a trace of iron salt. Indeed this reaction is the most sensitive known test for an iron salt. How, then, is this reaction catalyzed? In this case we do not need hydrogen peroxide; or if it is important it is formed spontaneously from the air. A ferric salt at once oxidizes cysteine to cystine. The reaction goes in the absence of air if only the iron is in the ferric state. There is at the moment a transitory formation of a violet or blue color. Now this color is very interesting since it shows that for a moment there is a ferric and a ferrous atom in the same molecule. Just where the iron unites with the cysteine, whether in the carboxyl or the SH group, is uncertain. Probably it is in the latter. One of the sulphurs is thereby oxidized to SOH, which then condenses with the other unoxidized cysteine molecule to make cystine. This oxidation is considered presently more at length.

3. *The action of catalase in respiration:*

The first of the respiratory enzymes to be considered is the diastase, *catalase.* There is a difference of opinion as to the rôle this plays in the cell. Hopkins (1923), for example, considers catalase as acting only in the one direction, namely, that of destroying hydrogen peroxide and he considers catalase as a substance which from its action on hydrogen peroxide indicates that "the influence of peroxides is at most of secondary importance" in biological oxidations. The idea that it may be acting normally in the opposite direction toward the synthesis of peroxide does not seem to have been considered. We might with equal right argue that, since the endoproteases normally destroy the proteins, therefore they can be playing no important rôle in the synthesis of the proteins. The evidence that catalase does play some important part in cell respiration is in brief the extraordinary parallelism between the content of catalase in tissue and the respiratory activity of the cell, and the further fact that the enzyme appears to be universally present in living things of all kinds.

Catalase is found in all cells so far examined and the catalase activity of cells goes closely parallel on the whole with their respiratory activity. The catalase increases with an increase in respiration and diminishes as the vitality or respiratory action of the cell diminishes. Thus there are two kinds of seeds of *Xanthium*, the cocklebur, which differ in their germinating behavior. The upper seeds have coats somewhat less pervious to oxygen than the lower, and require accordingly a somewhat higher pressure of oxygen for germination. These seeds can be made to germinate by rupture or removal of the oxygen-resisting membranes (Crocker). The upper seeds are also smaller. Their germination is delayed. The catalase content of the two kinds of seeds is quite different. The lower seeds show, when ground fine, 155 to 206 per cent greater catalase activity than the upper; and their respiratory consumption of oxygen is also greater than the upper. Furthermore, the catalase content and the respiratory activity increase together as the seeds germinate, both increasing as germination proceeds. Furthermore, as oxygen becomes available after the coats are broken, there is a marked increase in catalase. When the germinating power is poor so also is the catalase content (Shull and Davis, 1923).

Similar parallelisms of catalase activity with respiration in plants had been found previously by Appleman in 1916, and in sweet corn by the same author in 1918. Crocker and Harrington have compared with the same results the catalase and oxidase content of seeds with age, vitality, and respiration.

On the animal side similar striking parallelisms between cell respiration and catalase content of tissues have been established by Burge, who found that anaesthesia reduced the catalase content of tissues, and who has published a whole series of papers on this important subject. Atwood, 1922, found that treatment of wheat by formaldehyde affected the respiration and the catalase activity in a strictly parallel fashion.

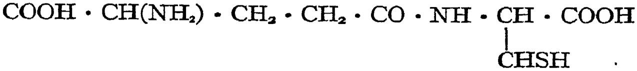
These investigations are so uniform in their results and occur so regularly in both plants and animals that there can be no doubt that in some manner or other catalase is very intimately related to the respiratory activity of cells.

We may summarize the action of catalase with the statement that, while its only known action is the catalysis of hydrogen peroxide to oxygen and water, its greatest use in the cell may possibly be in the opposite direction. Reasoning from the reversible action of enzymes, it may be that it greatly hastens the formation of ozonic acid and hydrogen peroxide from oxygen and water. While the equilibrium of this reaction certainly is reached when there is very little peroxide present, yet in the presence of reducing substances, which remove ozonic acid and peroxide as rapidly as they are formed, the catalysis of the reaction may be of great importance in respiration. The remarkable parallelism between the catalase content and respiratory activity shows certainly that catalase must be most intimately related to cell respiration as Burge (1917–23) and Crocker and Harrington (1918) and Appelman (1916) have maintained.

4. *The rôle of sulphur compounds in respiration:*

The possibility that sulphur compounds, and especially cysteine, might be playing a part in cell respiration was suggested by Heffter in 1907 and independently by the author in 1908. Heffter showed by means of a color reaction with nitroprusside of sodium that some tissues apparently contained cysteine or some other sulphur compound. The reducing powers of protoplasm were, the author thought, similar to those of cysteine. The whole matter has been recently continued by Hopkins (1921) with his discovery of a dipeptide consisting of glutamic acid and cysteine, which he has called glutathione. Cells which are old or dead, with little vitality, do not give the nitroprusside reaction or give it but faintly. This reaction, for example, is given by the fresh normal lens of the eye, but not by the cataract lens (Abderhalden, 1922). The author about the same time as Heffter, and quite independently, was working on the spontaneous oxidation of cysteine, thinking that it was a type of what was happening in living matter. He found, actually, that cysteine oxidized just under the same conditions as did protoplasm, that is, it was very sensitive to acids and alkalies and oxidized only with any speed at a H-ion concentration of about 10^{-8} N; moreover, it was tremendously catalyzed by a trace of iron; that the reaction was increased by very small amounts of arsenic and some other substances, and that it was poisoned in a remarkable way just as was protoplasm by traces of potassium cyanide and nitriles, and by such poisons as silver nitrate and mercury. It was not, however, interfered with by anaesthetics, in this respect differing from the vital reactions. The author concluded that these resemblances indicated that the respiration of cells had something to do with the sulphur and possibly with the conversion of cystine to cysteine as it exists in the protein molecule, but that there were other more important factors.

A year or two ago Hopkins in following this further succeeded in isolating from muscle and yeast extract the substance which gave the nitroprusside reaction. It was a dipeptide consisting of cysteine and glutaminic acid and he named it "glutathione." In a recent paper Hopkins has attempted to bring this substance which is of the composition:

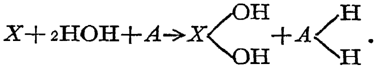


$$\mathrm{COOH\cdot CH(NH_2)\cdot CH_2\cdot CH_2\cdot CO\cdot NH\cdot \underset{\underset{\displaystyle CHSH}{|}}{CH}\cdot COOH}$$

Glutathione

into relation with protoplasmic respiration. For many oxidations to occur, it is necessary to have at hand a hydrogen acceptor. The oxidation, then, he thinks goes as follows under anaërobic conditions, X being a cell constituent, and A a hydrogen acceptor.

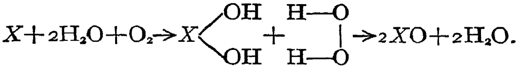
(1)



$$X+2\mathrm{HOH}+A\rightarrow X\!<\!\begin{matrix}\mathrm{OH}\\ \mathrm{OH}\end{matrix}+A\!<\!\begin{matrix}\mathrm{H}\\ \mathrm{H}\end{matrix}.$$

And under aërobic conditions,

(2)

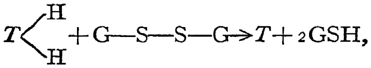


$$X+2\mathrm{H_2O}+\mathrm{O_2}\rightarrow X\!<\!\begin{matrix}\mathrm{OH}\\ \mathrm{OH}\end{matrix}+\begin{matrix}\mathrm{H{-}O}\\ \quad|\\ \mathrm{H{-}O}\end{matrix}\rightarrow 2X\mathrm{O}+2\mathrm{H_2O}.$$

Now, there is no doubt that hydrogen is at times set free in cells. Oxidations and reductions go hand in hand. Not only do aldehydes oxidize to acids, but at the same time they reduce to alcohols.

An oxidation by cystine (or diglutathione) might be produced as follows (Hopkins), where T is a tissue constituent not autoxidizable and G—S—S—G, is diglutathione

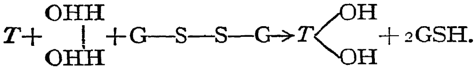
(1)



$$T\!<\!\begin{matrix}\mathrm{H}\\ \mathrm{H}\end{matrix}+\mathrm{G{-}S{-}S{-}G}\rightarrow T+2\mathrm{GSH},$$

or

(2)



$$T+\begin{matrix}\mathrm{OHH}\\ |\\ \mathrm{OHH}\end{matrix}+\mathrm{G{-}S{-}S{-}G}\rightarrow T\!<\!\begin{matrix}\mathrm{OH}\\ \mathrm{OH}\end{matrix}+2\mathrm{GSH}.$$

(3) $$2\mathrm{GSH}+\tfrac{1}{2}\mathrm{O_2}\rightarrow \mathrm{G{-}S{-}S{-}G}+\mathrm{H_2O}.$$

Glutathione behaves in all respects like cysteine but it has the advantage that it is soluble in the dithio state, that is, in the cystine state, and does not so easily precipitate out of solution. There seems to be no change in its other properties due to the union with glutamic acid.

What part, then, if any, does cysteine play in the cell respiration? The first suggestion which occurred to the author was that it acted as an autocatalytic agent which would set free active oxygen which would attack and oxidize any substance in the solution with it. Experiments were accordingly tried adding to the cysteine solution glucose, tyrosine, resorcine, and soap, and testing the rate of oxygen uptake and the amount taken up. To my surprise there was no change in the rate of oxidation or indeed any oxidation of the added substances due to the simultaneous oxidation of the cysteine. This observation was not published as the results were negative. Hopkins has repeated the same experiments with glutathione and with the same result. But he finally found one substance which was accelerated in its oxidation: this was an unsaturated fatty acid. Thus the glycerol ester of linoleic acid was oxidized. But linoleic acid is itself readily oxidized and takes up oxygen spontaneously at a rapid rate. Its oxidation is also autocatalyzed, like that of cysteine which is also catalyzed by the presence of some of the disulphide compound such as cystine, or the disulphide form of glutathione. Just how this is to be explained is uncertain, but Hopkins suggests that it may be due to an addition compound of the sulphydryl form and the disulphide.

This negative result shows that, whatever the nature of the oxidation of cysteine to cystine, it does not involve an increase in the active oxygen in the solution.

There is little doubt, however, that cysteine either in the free form or when combined probably plays a very important part in cell oxidations. Our knowledge in this direction we owe in large measure to Batelli and Stern (1911) and to Hopkins and his pupils. Batelli and Stern made the very interesting and pregnant observation that ground-up muscle of birds takes up oxygen from the air at a rapid rate. But if this muscle is washed with distilled water first, neither it nor the washing water will take up oxygen separately. But if the washings are added again to the muscle, then the muscle takes up oxygen just about as rapidly as before. It appears then that there is in muscle a water-soluble substance possibly attached to the outside of the cells, which is necessary for the taking up of the oxygen. This substance they did not identify but called it "pnein." Hopkins has shown that glutathione acts just like the pnein. If glutathione is added to washed muscle, the muscle again takes up oxygen, and a quantity of oxygen is taken up far in excess of that which can be taken up by the glutathione when the latter is converted to the disulphide form. Oxygen is consumed until all the available oxidizable substance in the tissue is oxidized. The glutathione itself undergoes no oxidation except that reversible one to the disulphide.

These very important observations indicate that cysteine, or glutathione, or some similar substance acts the part of an oxygen carrier to the elements of the tissues.

5. *The process of formation of carbon dioxide:*

In this connection the fermentation of glucose to produce alcohol and carbon dioxide is of interest. We here come to a new factor in respiration, namely, the rôle of the phosphates. All cells contain phosphates of which the rôle in the cell is by no means clear. Now, it has been found that these phosphates are of great importance in the fermentations of the sugars and that as a matter of fact the substance which is fermenting is the hexose diphosphate. Just how this is formed is uncertain, but there is no doubt that its formation is in some way an important preliminary to the fermentation of glucose by yeast.

The next step of the process appears to be the hydration of the hexose molecule. It is split into two molecules of carbon dioxide and two molecules of alcohol. This process of the hydrolytic splitting of the carbon chain is dealt with on page 62. By the hydrolytic decomposition of the chain acetaldehyde is first produced with carbon dioxide. This in its turn is a reduction and oxidation; that is, the terminal carbon is oxidized and the second one is reduced. The next step Neuberg thinks consists in the reduction of the acetaldehyde to form alcohol.

In this reaction the acetaldehyde acts the part of a hydrogen acceptor and may be an important element in cell respiration.

The enzyme, carboxylase, by setting free carbonic acid exposes the next carbon atom to the action of oxygen.

6. *Rôle of potassium:*

Another substance which is in some way concerned in the respiration of cells is the element potassium. It has been found that, in the presence of potassium hydrate, phloroglucin and similar substances undergo autoxidation better than with an equivalent amount of sodium hydrate. This indicates that the potassium salt is more easily oxidized than the sodium. There must be some reason for the preference cells have for potassium over sodium. The general richness of potassium in cells of widely different character indicates that this element must be concerned with some fundamental process or condition in the cell, and it is possible that that process is respiration. But just why it is favorable or what its real function is it is impossible to state.

In conclusion it may be said that knowledge of respiration is still so incomplete that no general synthesis is at present possible and we can do no more than call attention to some of the important factors which are interacting. Needless to say there are others which have not even been considered, among them the reaction of the cell, the rôle of oxidases, and so on.

XI. THE SYNTHETIC POWERS OF PROTOPLASM

Let us now examine the nature of the process of synthesis possessed by protoplasm. The simplest of the complex substances which are formed are the carbohydrates. We may begin with glucose. This can be synthesized by animals out of lactic acid, acetic acid, glycerine, aldehyde, and various amino acids, and it can be formed from carbon dioxide by plants under the influence of light. In this case we have a synthesis of a long carbon chain out of short carbon chains or even of single carbon atoms. The general equation can be written

$$6H_2O+6CO_2=C_6H_{12}O_6+3O_2.$$

Now while at first glance this does not appear to be a dehydration synthesis it is in reality such. It is a typical dehydration synthesis such as is at the basis of all cell life, and will throw light on all cell processes if we study it.

In this case we are fortunate that the synthesis does not require the action of living matter. All that is necessary, according to Baly, Heilbron, and Barker (1921), is that carbonic acid be illumined by ultra-violet light of the wavelengths 200 $\mu\mu$ and 250 $\mu\mu$. When that is done there is the formation of formaldehyde and oxygen in the method just suggested and afterward the condensation of the formaldehyde into carbohydrate. Let us take the second process first. Instead of using light we may produce the condensation with alkali also.

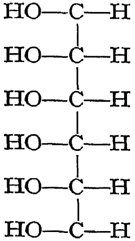
If a solution of formaldehyde, CH_2O, is made slightly alkaline, particularly by the addition of calcium hydrate, it is converted spontaneously into a syrup. The mixture is called α-acrose. Now what is the nature of this condensation? Obviously there is no use discussing that which occurs in living matter if we cannot first understand this. It looks at the first glance as if the condensation was not more than the condensation of several molecules of formaldehyde to form one of a carbohydrate as follows:

$$6CH_2O = C_6H_{12}O_6.$$

But let us look for a moment at the formaldehyde. According to Nef, and after him to Baly, Heilbron, and Barker, in the case of ultra-violet light, the formaldehyde at first is converted by alkali or light into a more reactive form in which the carbon is divalent. In this form it contains more energy since it is more unstable. The formula of this anakinetomeric formaldehyde may be written:

$$\mathrm{HO{-}C{-}H}.$$

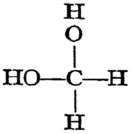
While in this form, it is certainly very reactive. The carbon has two unoccupied valences. We may suppose, on the theory of Bohr (1923), that one or more of the electron orbits have a quantum or several quanta of energy more than in the stable configuration of H_2CO. This active bivalent carbon is then supposed to condense with other groups of the same kind to form $C_3H_6O_3$ and other carbohydrates, $C_6H_{12}O_6$, etc.



$$\begin{array}{c}\mathrm{HO{-}C{-}H}\\ |\\ \mathrm{HO{-}C{-}H}\\ |\\ \mathrm{HO{-}C{-}H}\\ |\\ \mathrm{HO{-}C{-}H}\\ |\\ \mathrm{HO{-}C{-}H}\\ |\\ \mathrm{HO{-}C{-}H}\end{array}$$

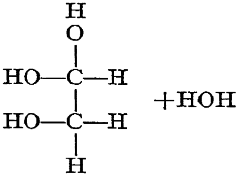
The two terminal carbons are thus left with three valences each. They divide between them a molecule of water, a hydroxyl going into the topmost carbon, and a hydrogen going into the lowermost carbon. This completes the carbohydrate. Or they unite tail to head to form the cyclic hexose, inosite.

But the essential nature of the synthesis as a dehydration synthesis, as it is in reality, may be brought out if we write the formula of active formaldehyde as follows:



$$\begin{array}{ccc} & \mathrm{H} & \\ & \mathrm{O} & \\ & | & \\ \mathrm{HO}— & \mathrm{C} & —\mathrm{H} \\ & | & \\ & \mathrm{H} & \end{array}$$

This carbon atom is now seen to be polar. The upper pole and the left one are oxidized since they have hydroxyls attached to them and hence are electropositive. The lower pole and the one to the right are united with hydrogen. They are reduced and hence electronegative. Now, if two of these molecules come together so that the positive pole of one is toward the negative valence of the other, they unite, water being eliminated between them, as follows:



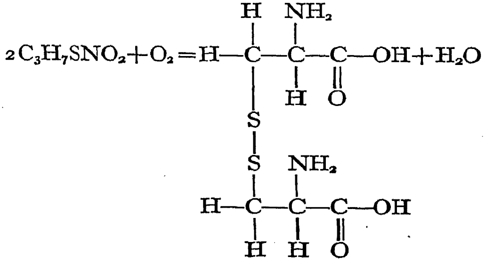
$$\begin{array}{cccc} & \mathrm{H} & & \\ & \mathrm{O} & & \\ & | & & \\ \mathrm{HO}— & \mathrm{C} & —\mathrm{H} & \\ & | & & +\mathrm{HOH} \\ \mathrm{HO}— & \mathrm{C} & —\mathrm{H} & \\ & | & & \\ & \mathrm{H} & & \end{array}$$

It will be observed that this group of two formaldehydes, glycolaldehyde, is like the original molecule, polar. That is, the upper end of the molecule has the valence oxidized so that the carbon at that point is electropositive; and the lower end of the molecule is electronegative, there being a hydrogen at that point. A molecule of water is eliminated between the two carbons. The condensation is hence a dehydration and we have a carbon chain built up in this way. The chain can in turn be ruptured again simply by hydration. It is clear from all the behavior of the aldehydes that they add and drop off water with great ease. The two carbon atoms of formaldehyde thus coming to lie close together, with positive to negative poles, the water is dropped from them in a way I don't understand, and they unite directly. And so we can go on adding always another formaldehyde molecule at the ends until the hexoses are formed. The process does not seem to go easily beyond the hexoses, since heptoses are rare, but pentoses and possibly tetroses are common. The cessation with the hexose is possibly owing to the formation of butylene oxide rings in the hexose molecule which are very stable and which, by the loss of water within the molecule, interfere with the loss of water at the ends and hence with further synthesis by dehydration. Certain it is that the hexoses hold a single molecule of water in the terminal carbon with a great deal of firmness. They do not, in other words, easily lose this water. They generally crystallize from solution with it as $C_6H_{12}O_6 \cdot H_2O$.

It is seen that this condensation is a typical synthesis by dehydration; that it goes spontaneously in the light as soon as the molecule of formaldehyde has absorbed a proper quantum of energy from the light and been converted into the unstable anakinetomeric form. It does not need any vital energy of any kind, or the existence of any vital force. Furthermore the molecule thus formed is tolerably stable, as long as the reaction of the medium remains neutral. The condensation is owing to the fact that two atoms come near together which are polar, that is, oxidized at one point and reduced at another. They combine as these opposite groups come together, water which has been occupying these positions being crowded out to one side. The union is due to the fact that the two electrons on the negative pole enter into the system of the other carbon also so that they are shared between the two atoms.

That this is the course of events is strongly indicated by the result of the addition of alkali to carbohydrate, especially to glucose, by which the reactivity of the glucose is similarly greatly increased. If the alkali is very weak the chain is not split readily, but there is a rearrangement of the terminal carbons, Nef states, with the formation of several different sugars, ketones being formed from aldehydes, an enol form and ethylene oxide being intermediate, and spontaneous condensation to a disaccharide takes place. But if the alkali is strong hydration occurs in the chain also, and the molecule is fragmented. This fragmentation is in the nature of a hydration. The water is put back in between the carbons, fragments containing two or three carbon atoms being produced; this is the reverse process to that just described for the synthesis of the sugar from formaldehyde in other words. Formic acid is produced by this fragmentation, carbonic acid, acetaldehyde, dioxyacetone, and other substances, including many acids, indicating that in some cases the hydroxyl has gone back where the hydrogen was before, thus oxidizing that carbon while the hydrogen went to the other, and thus reducing it to an alcohol.

Another dehydration synthesis of this same nature is that of cysteine to cystine. Here again we have the union of two similar atoms, one of which is oxidized, the other unoxidized. It is well known that the union is brought to pass by the action of oxygen. It is usually expressed as follows:



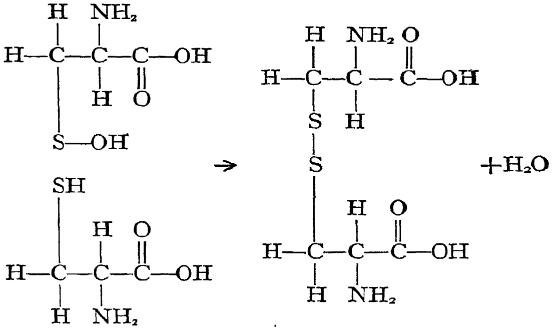
$$2C_3H_7SNO_2+O_2=\begin{array}{ccccccc}
 & & H & & NH_2 & & \\
 & & | & & | & & \\
H & - & C & - & C & - & C-OH+H_2O\\
 & & | & & | & & \| \\
 & & | & & H & & O\\
 & & S & & & & \\
 & & | & & & & \\
 & & S & & NH_2 & & \\
 & & | & & | & & \\
H & - & C & - & C & - & C-OH\\
 & & | & & | & & \| \\
 & & H & & H & & O
\end{array}$$

Probably, however, the reaction is as follows in steps,

(1) $C_3H_7SNO_2+O=C_3H_6S(OH)NO_2$

Hydroxycysteine

(2) $C_3H_6S(OH)NO_2+C_3H_6SH\cdot NO_2=C_6H_{12}S_2N_2O_4+H_2O$



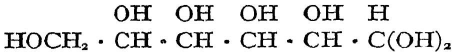
Cystine

The sulphur of one molecule is presumably first oxidized to form hydroxycysteine; a molecule of hydroxycysteine and one of cysteine then unite with the elimination of water. The alkylated sulfhydrates are known to be strong bases. Hence the upper molecule would probably dissociate $\overline{OH}$ and the lower H^+. It is a typical dehydration synthesis, and here again one atom of sulphur is oxidized one degree more than the other. Hence it is perhaps that, if cystine is boiled with sodium hydrate and lead oxide, only a portion of the sulphur, or approximately half, can be recovered as sulphide sulphur. The other sulphur atom has been oxidized. It is as a matter of fact on its way to form taurine. For this it is necessary to split off carboxyl and to add another atom or two of oxygen to the sulphur; that is, it is only necessary to take away the other electrons of the outer shell of the sulphur.

This dehydration synthesis, which there is every reason for believing to take place in the living cell, thus occurs equally well outside the cell. It needs no special vital activity. The condensation is an indirect result of an oxidized and unoxidized atom of the same kind being brought into juxtaposition. A preliminary oxidation is necessary for the synthesis.

Here again, also, the process is reversible. It can easily be reversed by reduction in an acid medium, by tin and hydrochloric acid. But it can also be reversed by alkalies alone, the cystine being reconverted into a molecule of cysteine and one of hydroxycysteine.

If we examine the other cases of dehydration syntheses they all have this in common, namely, they are the union of a group which is not so much oxidized with one which is more oxidized. Thus for example in the disaccharides, the union is brought to pass by the condensation of an aldehyde with an alcohol group. The former is oxidized one degree more than the other. Now, this union will take place slowly in the presence of acid or very dilute alkali (Nef). It is probable that the reaction depends on the conversion of the aldehyde group into its active form. It is not at all probable that this is the butylene oxide form of the sugars. Indeed, this is in the highest degree unlikely. It is more probable since these are the most stable forms that it is first necessary to convert the inactive butylene form into the active form. These active forms may be regarded as gamma sugars, if the reactive form of sugars be called gamma sugars. It is certainly the anakinetomeric form of the sugar, the energy-rich form, which has this power of spontaneous condensation. From what has been said about formaldehyde it is clear that the unstable reactive form of the sugar which will condense so readily with an alcohol that it goes on in an aqueous medium and at room temperature in plants with great speed is probably the hydrated aldehyde. The alkali or acid hydrates the sugar, rupturing the butylene oxide ring.



$$HOCH_2 \cdot \overset{OH}{CH} \cdot \overset{OH}{CH} \cdot \overset{OH}{CH} \cdot \overset{OH}{CH} \cdot \overset{H}{C}(OH)_2$$

It is this form which adds at once without difficulty the hydrogen group and one sugar group. After this group is formed the molecule may secondarily fall into the stable form of the butylene or amylene oxide ring.

Here again the union to form the ring is between the oxidized and the partially oxidized group. We thus see that the preliminary to the condensation is the acquiring of more energy by the sugar molecule. This accounts for the fact that these unions cannot take place if oxygen is absent, for in that case the energy is less easily obtained.

A similar state of affairs is seen in the condensation of the fats to form ester. In this case we have the union of a carboxyl group, which is the limit of oxidation consonant with the carbon remaining in a chain with the partially oxidized alcohol group. Here again the two groups condense. But again this condensation takes place with the greatest ease in living matter, but not so outside. It is certain that here again we must be dealing in living matter with a very reactive form of one of the two constituents. This is shown by the fact that the splitting by means of lipase goes to completion in an aqueous medium; if one wishes to get this synthesis reversed it is necessary to work in a medium as free as possible from water. This indicates that the active form may be one which has lost water. This might be the glycerol. The terminal carbon possibly loses water owing to union with some element of the protoplasm. This converts it to the bivalent form, which might add the carboxyl group without difficulty. When lipase splits the glycerol off, it reverts at once to the inactive form where it is hydrated. Each atom perhaps is thus dehydrated in turn.

There is another case of this same sort of synthesis, namely the synthesis of the proteins from the amino acids. This is in all respects like that of the carbohydrates or rather that of the formation of an ester. The amino group has the significance in oxidation of a hydroxyl group. The amino acids may, indeed, be treated like the hydroxyl acids. There is then a union of the oxidized group with the reduced or less oxidized group of the alpha carbon. Again we have to ask how this is carried through. The answer to this question is the same as with the fats. It is unknown. The fact that the digestive enzymes such as trypsin reduce the proteins practically to the state of amino acids or at least of tripeptides and amino acids and that the synthesis is not reversible proves that the state of the amino acids in the protein molecule must be an unstable one and cannot be the same as the stable state which the acids have after they are split off. We have then to ask what this unstable state can be? How is it possible for the amino acids to exist in an unstable form. Does this instability concern the carboxyl group or the amino group? It is clear that here we are in difficulties. The explanation which seemed probable in the case of the alcohols such as glycerol cannot be correct here. For if the amino acid lost ammonia so as to become bivalent carbon in the alpha position, there would be no amino group with which the carboxyl group could unite. Nor are we in a better position with the imino group.

There is one other thing in this connection which is worth considering and that is the action of carboxylase. It is the diastase which separates carboxyl from the amino acids and leaves the amine. This is also a typical hydrolysis. It is as follows:

$$CH_3—CHNH_2—COOH+H_2O \rightarrow CH_3CH_2NH_2+H_2CO_3.$$

If this process were reversible we ought from the amines to make the amino acids by the addition of carbonic acid. So far as the author is aware this has not yet been accomplished by the action of carboxylase.

This paragraph on dehydration syntheses may be summed up as follows: In all cases in which the process can be studied it consists in the endowing of one molecule of an extra amount of energy. The addition of this energy by changing an atomic orbit or the configuration of the molecule makes it more reactive and unstable. The greater reactivity leads to the condensation of the two molecules with the elimination of some of the energy which escapes as heat. There are then two questions which we have to consider: How is this energy raised? What is the origin of it and what are the particular forms of the reactive molecules which transitorily exist? For it unfortunately happens that the molecules which accumulate in cells are always those which are stable; whereas the molecules which exist in living matter and endow it with its peculiar properties are the unstable ones. All we can at present do is to guess at the reactive forms.

XII. HYDROLYTIC DECOMPOSITIONS IN CELLS. THE HYDRATION OF THE CELL CONSTITUENTS

Under this heading we take up some very important chemical processes, for a large part of the chemical changes in cells consists of the hydrolytic decompositions going on in them. We have already referred to these changes in connection with the opposite change, that of dehydration syntheses. It was pointed out that the latter went on only in the presence of oxygen, and while the cell was intact in its structure. The hydrolyses, however, do not depend upon cell structure or upon oxygen. They go on just as readily in the absence of oxygen as in its presence. By hydrolyses one means reactions in which a separation (lysis) of two or more atoms formerly united into a molecule is brought about by the entrance of the elements of water. A hydrogen and a hydroxyl ion enter. By this a decomposition is brought about, a loosening by means of water, as the word means. All the processes of digestion of foods as well as the splitting of carbon chains are hydrolytic decompositions.

These decompositions are produced in cells by the action of special catalytic substances called diastases or enzymes: proteases, lipases, nucleases, zymases, amylases, carboxylases, glucosidases, and many others. Every complex substance in a cell is accompanied in many if not in all cases by a hydrolytic enzyme which acts upon it. Thus every cell which contains starch has always an enzyme amylase which will digest the starch. It is often difficult to demonstrate the presence of these enzymes and they are often overlooked, but they can usually be shown to be present. Sometimes a preliminary digestion is necessary to set them free. They seem to be covered up or concealed in a more complex molecule. Such a concealed state of an enzyme is called a zymogen.

It will repay a moment's digression to consider these zymogens. They have long been a favorite matter of discussion of cytologists. The granules in the cells of the stomach are called zymogen granules. In the pancreas, and indeed in all glands in which enzymes are secreted, similar granules are seen, which, since the quantity of diastase recoverable from the gland goes roughly parallel to the amount of these granules, are generally assumed to be zymogen granules. Experiments show that usually the enzyme, or diastase, is secreted from the cell only partly in the active form or not at all in the active form, but in the form of the zymogen. Thus we have pepsinogen from the stomach, characterized chemically by the fact that it is less sensitive to alkalies than the free pepsin; from the pancreas we have the inactive trypsinogen. This is set free or uncovered by the action of the diastase of the intestine, called enterokinase. Another instance is given by the lipase of the pancreas which is activated by the bile, and possibly by other agents. Another similar example is given by the saliva of the parotid of the horse. This as secreted by the gland is inactive on starch, but is activated by some other of the liquids of the mouth or by the bacteria. It seems, then, that there may be enzymes present but in an inactive form, and this inactive form is rendered active by the action of a preliminary hydrolysis by some other enzyme, by acid, by alkali, or by water. It is hard then to decide definitely that an enzyme is absent from a cell when we get no reaction for it, for it may be there in an inactive form.

An extremely interesting example of this sort has recently been found by W. M. Billing, of the William S. Merrell Company's biochemical laboratory. The results are yet unpublished, but Mr. Billing has very kindly allowed me to refer to them here. Billing, working on crotalin, the active principle of the rattlesnake venom, made a most interesting discovery. This poison, which others have suggested acted like an enzyme or diastase, but which no one had been able to show did so act, has an action of a very striking kind on the fibrinogen of the blood. It digests or peptizes it or changes it so that it disappears. But the fact which is even more interesting is its action on tissue fibrinogen, the substance called cytozyme, or thrombokinase, by some. If one injects into a guinea pig a sublethal dose of crotalin which has been mixed with some tissue fibrinogen, which in itself is quite harmless, the toxicity of the mixture is greatly enhanced, but for a brief period only. During this period a very small part of the lethal dose will kill. This shows almost certainly that tissue fibrinogen which is a very complex substance consisting of a phospholipin united with a protein and which is in so coarse a state of dispersion that it will not pass through a porcelain bougie, or even through fairly fine filter paper, contains within its molecule an enzyme which is not ordinarily in evidence, being covered up in some way, but which acts presumably as does the crotalin and peptizes the fibrinogen of the blood. This substance crotalin is in truth an enzyme. In fact many of our proteins may have enzymes concealed in them. This is a matter which will require further investigation, but it indicates a possible method of attack on the problem of immunity which Mr. Billing and his co-workers are carrying on. In other words, there may be more in a complex protein than appears at first glance.

To return now to the process of hydrolysis which is carried on by these hydrolytic enzymes. They are presumably the same agents which cause the synthesis by dehydration, or rather which catalyze that synthesis. How then shall we explain the fact that the synthesis depends on the intactness of the cell and that respiration is necessary for it, whereas the hydrolysis does not so depend; and further that all attempts to synthesize by means of these enzymes outside the cell have been unsuccessful except in a few cases and under circumstances quite different from those of the cell? For example, the case of lipase may be cited. This does indeed cause some synthesis if mixed with glycerine and fatty acid, but when in a watery and dilute solution such as prevails in the cell the action is in the direction of hydrolysis and goes practically to completion.

The explanation of the whole matter is simple in view of what has gone before. Neither hydration nor dehydration in itself involves much energy change; that is, there is very little energy set free on the hydrolysis of a protein or a polysaccharide. There does seem to be a little heat freed, but it is not much. But as we have seen, the substance which is about to be dehydrated and synthesized must have its energy content raised. It must be converted into the anakinetic form. This is accomplished by combining it with the protoplasm so that it can receive some of the energy from oxygen. It is here that oxygen is necessary. The diastase cannot synthesize the inactive form into the polysaccharide, neutral fat, protein, nuclein, etc. It can only catalyze the synthesis of the active form. It is for this reason that the synthesis, the dehydration, will only occur in the presence of oxygen, when the cell is respiring and when the cell structure is intact. All of these things are necessary not for the reversal of the enzyme action, but to form the anakinetomeric form of the molecule which is to be condensed and of which the condensation is accelerated by the enzyme. It is easy to see on the basis of the action of anaesthetics just sketched why anaesthesia checks the synthesis of the cell as well as all its other powers except the hydrolysis. The anaesthetic by occupying the oxygen receptors or preventing oxidation in other ways prevents the formation of the anakinetomeric forms of the molecules.

We have now a satisfactory explanation of that which has for so long been a puzzle. So far as I am aware there are no facts which are not reconcilable with this interpretation, although a long road remains for us to go over before we can be more precise in the matter and state positively just where and how the union takes place between the amino acid, or other substance to be condensed, and the protoplasmic molecule, and where the oxygen unites. These are matters to be followed out and no doubt they will when discovered throw light on many other things as well and very likely modify the general theory sketched here. But some kind of a picture or working hypothesis to account for these facts is necessary, and it is for that reason I have sketched it here.

The hydrolysis of the complex compounds does not depend upon energy supply. It is downhill so far as that is concerned. Each molecule of the hydrolysate as fast as it is set free from the complex, in so far as it has not already reverted to the inactive form while already in union, proceeds to pass at once to that form. And as this form cannot be resynthesized by the enzyme, the action appears to be irreversible. Hydrolysis proceeds without vitality; synthesis depends upon it.

In considering this hydrolysis, attention may be directed also to the first stage of the process, namely, what is called the hydration of colloids. This brings up again a most intensely interesting and obscure problem, namely, the condition of the water in cells and its rôle there. The water in protoplasm is an essential part of it. It is held there in peculiar condition. It is generally stated that the molecules are hydrated so that there is bound and free water. Dubois has studied this question. There is no doubt that the activity of cells of all kinds is dependent upon the amount of water in the cell and also no doubt upon its condition there. One of the earliest facts discovered about chemical stimulation was the discovery, for example, that the motor nerves of frogs may be stimulated by depriving them of water. Put a sciatic nerve of a frog in a strong solution of almost any fairly indifferent salt or non-electrolyte, and it generates nerve impulses. This fact has never been explained. How are these impulses generated? That is the question. Why should drying a nerve cause the discharge of its energy? The respiration of the nerve is also increased as is shown by the output of carbon dioxide under these circumstances (Tashiro and Riggs). The author explained this stimulation as due to a change of the colloidal state of the nerve. But this is very indefinite. I have seen nerves dried so gradually that they gave off no impulses, but they were becoming so irritable that at last the slightest jar would cause an explosive discharge of all their energy. Therafter they became entirely non-irritable, but would recover by being placed in physiological salt again.

For medicine this is a very important problem. The problem of oedema is bound up with it. The state of the water in protoplasm must be elucidated.

Some light in this field has been obtained by Fischer in a long series of valuable studies of the hydration capacities of soaps. What Fischer really studied was the amount of water a soap jelly would hold and still be a jelly. He made the ammonium, potassium, and sodium, and other salts of the various fatty acids and determined the concentration of soap necessary to form a jelly at room temperature. He found that the amount of water which could be held depended upon two things, first upon the length of the carbon chain of the soap used; and second upon the state of saturation of those chains. The amount of water which could be held was an exponential curve. In reality there is probably but the one factor here, namely, the length of the carbon chain; that is, far fewer molecules are necessary to make a gel with stearic acid $C_{18}H_{36}O_2$ than with caproic acid $C_6H_{12}O_2$. One can still make a solid gel containing less than 1 per cent of soap in a long carbon chain of saturated character, such as stearic acid, $C_{18}H_{36}O_2$, or arachidic acid, $C_{24}H_{48}O_2$. Oleic acid soaps, however, will not form gels, except in much greater concentration, although the number of carbon atoms is the same as that of stearic acid. The reason for this was not given by Fischer. But it may be that since the molecule is loose at the double bond, the molecule probably bends over at this point and the acid is in reality only as long as the nonylic acid, C_9 acid, since the double bond of oleic acid is in the middle.

Why, then, do the longer chains hold more water, or make a solid soap? It is quite possible that there is a molecule of water loosely attached between each pair of carbons. A C_{18} acid, like stearic, would thus hold to itself seventeen molecules of water. It will be recalled that these carbon chains are built up by a process of dehydration (p. 57). Probably there is always a tendency of the water to go back in, and water will be loosely held at these points. Something of the sort is almost certainly the case in glucose, which binds five molecules of water. Between each two carbon atoms, then, of all substances water may be bound, and possibly this is part of the way water is held in protoplasm. At any other union of a similar nature formed by dehydration a weak affinity for water will exist sufficient to hold the water.

But this does not explain why potassium salts form soft soaps and sodium hard. Fischer has interpreted this as meaning that the potassium salts are the more hydrated. This seems a not improbable explanation. It may be that in these chains the entrance of the water has gone so far as to reduce the rigidity of the chains, but this is pure conjecture. However, the fact that potassium favors the taking up of water by protoplasm, whereas sodium favors it less and calcium still less, would indicate some such action. Usually proteins swell more in potassium salts than they do in sodium. This probably means a greater degree of hydration.

Now it may be, although nothing definite can be said on the subject, that the presence of the hydrolytic enzymes, whose action is to decompose the molecule by complete hydration, favors the hydration of the protoplasmic colloids. This is a matter for further investigation. It might be thought that this action of potassium in favoring hydration might be brought into relation with the action of potassium in checking the stimulation of nerves by salts; but unfortunately the action of calcium is in the same direction as potassium, as regards stimulation, but there is no evidence that it favors hydration, but rather the reverse. We have come, therefore, to an impasse in this direction. The action of salts on irritability remain difficult to explain unless the colloids of the motor nerve are electropositive, as the author long ago suggested. The condition of the water in cells and in gels must be determined before we can go farther in this direction. Patrick has studied the condition of the water in silica gel. Here the gel appears to be made of micellae. On heating, the water comes off easily without any sharp points of inflection of the curve except for the removal of the last few per cent. This last water appears to be firmly and chemically bound, and is removed only by heating to several hundred degrees. When it is gone the gel is irreversible, and has lost its adsorbent properties. The gel seems to consist of micellae with water bound to them, making a very finely divided system in the interstices of which there is water held by capillary forces, or cohesion. A similar state of affairs exists according to McBain in soap gels.

XIII. THE ELECTRICAL PHENOMENA OF PROTOPLASM

The electrical phenomena of protoplasm, the current of action, the current of rest, the blaze current of Waller (1903), the injury current—these have been considered elsewhere (see p. 193). At this place I wish only to bring them into connection with the chemical constitution and character of the cell. These electrical phenomena have been known since the days of Galvani, their discoverer. They are among the most fundamental of the phenomena of the cell. That they are correlated with the life of the cell and the persistence of the irritability of the cell is well established. A. Waller has called the "blaze current," which even such things as seeds show, the electrical sign of life, since as soon as death comes on the electrical response is lost. Recently J. C. Waller has extended his father's observations and shown that if one places an electrode on two spots of a green leaf and shades one of the electrodes, the other electrode, that which is in the light, becomes electronegative to the first. There is a perfectly definite reaction when the light falls on the leaf analogous to that which occurs when light falls on the retina. It is not even necessary to place one electrode in darkness. If a variegated leaf be taken and one electrode be placed on the green part of the leaf and one on the white, the electrode on the green will become negative to the other when light falls equally on both. This shows that for the perception of light by the leaf chlorophyll is necessary. The reaction will no longer occur if the leaf is dead or if it is anaesthetized.

Always, therefore, in plants as well as animals the portion of a tissue or the portion of a leaf which is undergoing the more vigorous respiration, which is in the more vigorous and reactive state, or which is reacting more, becomes negative to the part which is less reactive. If a cell be injured, it will be found that the injured portion is negative to the uninjured part. This is a general law of protoplasm, and for it we seek an explanation. What is the origin of these electrical currents?

The various hypotheses to account for this current will be found elsewhere. I cannot go into them here. They differ according to the view of the nature of irritability which the author may have. Thus it has happened that in these recent years, for the past fifteen in fact, since irritability has been so generally and so erroneously referred to a state of permeability of the membranes of the cell, electrical phenomena had also to be so referred. All kinds of crude and incorrect guesses have been made in an attempt to refer the current to the permeability of cell membranes. The energy relationships have been entirely neglected, and the great fundamental fact that life is essentially respiration, an oxidation, and that this is the source of all its energy has been quite generally overlooked. A change in resistance to the passage of an electric current has also been assumed to mean that the permeability or ease of passage of ions through the cell membrane was the sole explanation. Thus the subject has been successfully camouflaged by a terminology of which permeability is the commonest recurring word. The attempt has been made to explain the electrical current of cells on the basis that it is a diffusion current and that only conductors of the second class, that is, liquid conductors, were concerned in it.

The futility of this view should have been apparent. Were it true we should have had a permanent source of energy without the expenditure of energy. All that we would have to do would be to connect up a diffusion chain and have an endless source of power. We might run a sewing machine without the expenditure of any energy at all. Now it is self-evident that there must be a source of this energy of the current. What is that source? It cannot possibly be a difference of concentration of ions. Such a source would be quickly exhausted even if it existed. Of its existence there has never been any proof. It makes no difference, for example, with what your electrodes touching the cell are impregnated, whether with sodium chloride or with other salts found in living matter and at the same or higher concentrations than they are in muscle or nerve, the current goes just the same. Evidently diffusion has little or nothing to do with it, except to carry the current which is generated in some other manner.

The living cell is in fact a battery. It is not a diffusion chain. All living things are batteries. In some forms of living things such as the electrical eel or torpedo this fact is so clearly apparent as to need no discussion. These animals generate a strong current at a good voltage. They have evidently a battery. Now, in every battery there is and must be somewhere a conductor of the first class, that is, there must be a metal in it somewhere. The current in all batteries is produced by the fact that some metal is being oxidized at a different rate at the two poles. Thus we can make a battery by taking a zinc wire and dipping the two ends in acid of different concentration, the two acids being in contact. In this case one end of the wire is being oxidized faster than the other end and as a result there is a current in the wire and through the solution. As each zinc atom is oxidized and goes off into the solution, like so much carbon dioxide or exhaled air, a new unoxidized atom remains behind ready in its turn to be oxidized. If we were able to keep on adding zinc at the one end and having it oxidized into the ionic form at the other, we should have a regular metabolism of zinc. There would be a catabolism at one end and an anabolism at the other. In other words, every battery has a metabolism of its own; and it is a zinc or copper metabolism usually. Furthermore the source of the current is always an oxidation. At one plate oxidation is taking place, at the other a reduction.

To make a battery we must have a system of this kind. We must have a conductor of the first class, that is, a substance which acts like a metal, and this conductor must be undergoing metabolism at a different rate at the two ends. There must be something of the same sort in a cell, but it puzzled me for a long time to imagine what and where it was. The interesting thing is that the conductor of the first class need not be long. It may be very minute. A piece of wire a millimeter or less in length will be enough provided only that the two ends are in a different state of activity, that they are in two different solutions one of which has a reducing, the other an oxidizing, action; or if the two solutions are unequally supplied with oxygen, if this is the oxidizing agent employed.

It finally occurred to me that the conductor of the first class in cells must be graphite. This, as is well known, can function as a metal in making a battery. It is actually a first-class conductor. It conducts like a metal. Now, if we had a rod of graphite so placed that one end of the graphite was being oxidized faster than the other, one being in a stronger oxidizing fluid than the other, there would be a current through the graphite in a definite direction. The next step was easy. It was only necessary to suppose that this rod of graphite was so arranged in the membrane of the cell that the one end was in the inside the cell, the other end on the outside. It is well known that there is a dearth of oxygen in the inside of cells. This end of the rod would then be in a condition of less oxidation than the outside, which is in an atmosphere of oxygen and in a place where the researches of Hopkins and his students indicate that oxidation is occurring.

But where was the rod of graphite? Again I was puzzled until the obvious answer occurred to me. It is of course some molecule made up of a chain of carbon atoms. Such a molecule is nothing else than a rod of graphite on a very minute and highly compressed scale. I have never been able to find this obvious idea expressed in the literature. I have never found any chemist who stated that the aliphatic carbon compounds must be conductors of the first class and resemble rods of graphite. But that they must so act it seems to me is certain.

There remained, then, only one thing to do. These rods must be oriented in the surface so that their ends are inward and outward, that is, they must have a palisade arrangement in the surface. They need not be exactly this. They might bend over in the surface, but the two ends must be one out, and the other in. But this is the very arrangement that Hardy, Langmuir, and Harkins had independently attributed to the molecules of fatty acids in a film separating oil and water.

There is a second necessity if such an arrangement is to explain animal electricity. The molecules cannot be conductors sideways; that is, there must be no metallic conduction from molecule to molecule. This assumption is necessary to explain nervous conduction. This necessity is provided for by such a palisaded arrangement of molecules. There would be no side conduction. It is not necessary for the molecules to be those of soaps or fats. Any other carbon chains, such as those of the proteins, will do. It may be, for example, that there is one molecule extending from the outside to the interior of the cell, having other molecules attached to it sideways such as cholesterol, proteins, etc., to make the membrane. The only essential is that there shall be no metallic conduction sideways in the membrane from molecule to molecule, and that there shall be metallic conduction from the outside of the membrane in. One side of this membrane, and I have assumed that it is the outer side, is oxidizing faster than the other. As each successive carbon atom on the outer end of the carbon chains is oxidized to carbonic acid, it is hydrolyzed off by carboxylase, leaving the next carbon atom of the chain either as an alcohol or aldehyde, ready for oxidation in its turn. The condition is entirely analogous to the condition in a battery.

It is clear that these conductors of the first class, namely, aliphatic carbon molecules, are not confined to the membrane of the cell, but that they exist elsewhere in the cell. In fact in the nuclear membrane something similar may exist. There also the molecules may be organized or oriented and the nucleus also may be a battery, the source of a difference of potential. There may, indeed, be something analogous to a current of action passing round and round the nuclear membrane all the time, just as with every transmission of an impulse there is a current of action running down the nerve. Imagine that we had a nerve arranged in a circle, the two ends being in functional union, the impulse once started would go on and on, around the circuit as long as there was oxidizable substance to sustain it. I am far from saying that this is the case, in a nucleus, but it is not an impossibility. We see something very analogous in the ceaseless circulation of the protoplasm of *Nitella* and other cells, or the impulse passing around the strip of a medusa bell. I have no doubt that if fine enough electrodes could be devised and placed on the right spots of the *Nitella* cell we should find that there was a wave of current which swept periodically over the cell corresponding to the rotational movement of the protoplasm.

We see, therefore, that the source of the electrical energy of cells must be just the same as the source of all their other energy, as the energy of light or heat or motion. It is oxidation. Every oxidation involves the passage of positive charges from the oxidizing to the oxidized body, or the movement of negative charges in the opposite direction. It is generally the latter. Every oxidation and reduction is hence an electric current; and every electric current is the expression of the fact that an oxidation is occurring in some part of the circuit and a reduction elsewhere. Every time an atom receives an electrical charge, a negative electron, it is reduced, and every time the electron leaves it, the atom is oxidized. This is what oxidation and reduction are.

It is curious to reflect that the nucleus may be a battery of this kind. Or that other similar circuits may exist in the cell. It recalls the conception of Rignano, which at first seemed quite impossible, that the nucleus acted like a storage battery and that impulses coming into the cell left some kind of an impress on the nuclear wall by which he believed it remembered, and these deposited memories modified the subsequent behavior of the cell. Crile (1923) has compared the nucleus to the brain of a cell, and the brain of man to his nucleus. He has suggested that these cell and nuclear membranes act the part of the leaves of a condenser, and this is not at all impossible. There is something very attractive about this idea; and recently a Dutch physiologist has shown that the electrical resistance of the body behaved exactly as a series of condensers and resistances behaved.

Let us now look into this matter a little farther. Let us suppose that we have on the outside of our membrane a union between oxygen and the graphite rod similar to that between oxygen and a molecule of hemoglobin. For some reason, possibly owing to bad conduction at some point, the oxygen molecule when it unites with hemoglobin gives up but a small proportion of its energy to it. The reaction $O_2 + Hb \rightarrow HbO_2$ liberates but little heat. For a gram of Hb uniting with oxygen it is only 1.85 calories, according to Hill and Barcroft. Now let us imagine that a fine electrode is placed on the oxygen thus united to hemoglobin, and another electrode at the other end of the hemoglobin molecule. What will happen at the instant when in some manner or other the oxygen gives up its energy (if it could give it up) to the hemoglobin molecule?

It is obvious that at this moment one or two negative charges pass from some point in the hemoglobin molecule, perhaps the iron atom, to the oxygen molecule. Or in another way of putting it, the oxygen gives up a positive charge to the hemoglobin, becoming itself negative. It will be seen that an electric current will pass from the oxygen to the hemoglobin (the electric current being always regarded as that of positive electricity). We would observe in the galvanometer connected with our electrodes a sudden deflection in such a direction as to indicate a momentary current passing from the oxygen-free end of the hemoglobin molecule to the oxygen through the external or galvanometer circuit. The outer end of the molecule where the oxygen is will appear to be negative to the other end.

The same thing is very likely true of the cell. Imagine now that the membrane contains and is partially composed of conductors of the first class as I have suggested. The oxygen is supposed to make a molecular union with the exterior ends of the oriented membrane molecules in the way brought out in considering respiration. The oxygen combines with what may be called the oxygen receptors. By this combination an unstable irritable substance is formed as already sketched. This is the loaded pistol as it were. The energy is there ready to flow, but it has only flowed in small part. Now put an electrode over the outer end of one or more of these oxygenated conductors, on one of these anakinetomeres, on one of these living molecules in other words, and place the other electrode in connection with the inside of the membrane through an electrolyte solution. Let us now stimulate this membrane so that the energy passes. There will be a current inward through the membrane and the circuit will be closed in part through the galvanometer. The electrode over the oxygen will become negative to that one in contact with the inside of the membrane. We observe what is called a current of action, or a negative variation. Since always every form of stimulus whatever its nature produces this flow of energy, we find that the stimulated portion, that is, the portion where this energy is being set free and chemical change is occurring in consequence will be negative to the other. It must be so on this hypothesis. Active protoplasm is then always necessarily negative to resting.

In other words, the electrical phenomena of protoplasm are simply the phenomena which happen within every molecule in every oxidation. There is nothing peculiar about the electrical phenomena of protoplasm. The only peculiarity is that protoplasm is so organized that these intramolecular phenomena become obvious extra molecularly. It is just like the magnetism of iron. No one would have guessed that it existed if it had not happened that by the organization of the atoms of certain kinds of iron this magnetic flux which was happening in every iron atom and presumably in every atom of every kind became visible on a scale more than atomic. So it is with the electrical phenomena of protoplasm. The current of action is nothing else than that current which occurs within every molecule when it is oxidized. Respiration and current of action—these are two words for the same thing. We cannot have respiration without an electrical current; and we cannot have an electric current without an oxidation and reduction.

The action of anaesthetics in reducing the respiration and in stopping coincidently the irritability of the cell can now be understood. The author has suggested that the anaesthetic displaces the oxygen from its receptors and thus forms a non-irritable anaesthetic-protoplasm compound. This brings respiration to a standstill, and as respiration and the electrical phenomena are the same, the electrical phenomena stop also. The following picture of the compound may help in giving a clear-cut conception. The various anaesthetics are then nothing else than drugs which can reversibly occupy the oxygen receptors of the cell.

The anaesthetics always stimulate at first when in very small amounts and then depress, and usually after one or two exposures the preliminary stimulation is lost and depression comes from the start. Furthermore very small amounts act as stimulants to growth, respiration, and other powers of the cell for a considerable period. A possible explanation of these facts has been given by the author. The first effect of the anaesthetic is to reduce the amount of partially oxidized protoplasm as the anaesthetic occupies some of the oxygen receptors. This produces the same effect as a beginning asphyxia. There is at once an active reaction on the part of the cell to get more oxygen. This reaction is well known in physiology. It is part of the general mechanism of adaptation by which protoplasm seeks to regain its balance and rise superior to circumstances. Now, if the anaesthetic comes in slowly, this adaptive reaction throws open for the time being far more oxygen receptors than the anaesthetic can occupy, and there is for a brief period the formation of more of the oxygen compound than is normal. Since it is this oxygen compound which contains the energy, there is for the time being more of the active, irritable, or living matter in the cell than normal. The irritability of the cell is hence temporarily enhanced and all the processes are stimulated. If, however, the anaesthetic is coming in too rapidly, or if the reserve powers of the cell are exhausted, as after several anaesthesias, then there will be no preliminary stimulation. This accounts satisfactorily for the preliminary stimulation and for the adjustment to poisons which Child has found. The more vigorous and active, the greater the reserve of the protoplasm, the more certain it is to adjust itself to small amounts of anaesthetics, cyanides, and other poisons. But also the more certain is it to be killed by larger doses. If protoplasm is less reactive, it is less susceptible to small doses, but its powers of adjustment to still smaller doses are reduced.

The after-stimulation which cells show on coming out of the anaesthetic are also explicable. As the anaesthetic escapes from the cell there is left an unusual number of oxygen receptors to which the oxygen unites, thus forming a larger than normal amount of irritable, or anakinetomeric substance. This raises the irritability above the normal and causes the post-anaesthetic hyperexcitability.

This explanation does not preclude the fact that the anaesthetic may be acting possibly in other ways also, such as an alteration in the permeability of the membrane to certain substances or a change in the viscosity of the cell. But the main action is no doubt one on the respiration of the cell. The essential matter is that the respiration of the cell is suppressed or diminished by the anaesthetic during anaesthesia. A substance like nitrous oxide may be acting in part, for example, by a slight alteration in the H-ion concentration since it is certain that a change in the direction of a rise in H ions slows respiration.

The following rough diagram (Fig. 1) presents in a schematized form the conception of the nature of the current of action and also of the nature of the conduction of a stimulus such as a nerve impulse.

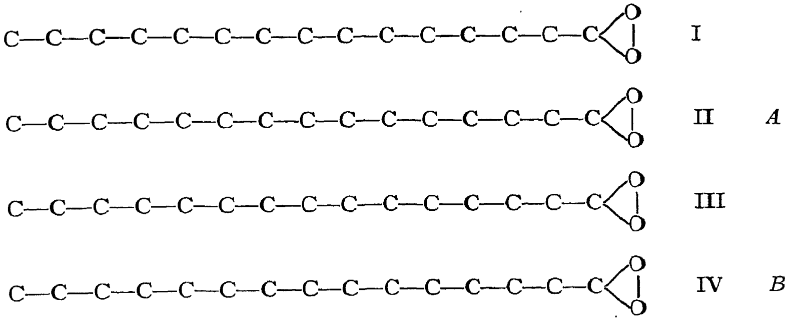


FIG. 1

This is the schematized membrane of carbon compounds with oxygen receptors occupied with oxygen molecules on the periphery. The oxygen has united with the terminal carbon groups but has as yet delivered but a small part of its energy. To enable it to give up the rest a shock of some kind is necessary. This partially oxidized substance is what is known as the irritable substance. Now let any form of a stimulus be given; the oxygen passes over its positive charge, that is, it receives the negative electrons from some point in the interior of the cell. One electrode we place over molecule number II at *A*, and the other is over molecule number IV at *B*. At the moment molecule under *A* gives over its charge and the act of respiration is completed, a current flows from *B* to *A* through the external circuit. The current is closed through the neighboring molecules and electrolytes. This sudden current acts as a stimulus to cause molecule III to discharge and this in turn sets off molecule IV. Thus the impulse passes down the membrane. As molecule IV respires it becomes negative and A is then positive to it. After this oxidation the terminal carbon atom may escape as carbon dioxide; and the next carbon atom is then exposed in its place. Oxygen re-enters this carbon and the irritable substance is so reconstituted. Thus oxygen is taken up and carbon dioxide is given off during the period of repair, rather than at the moment of oxidation. This has been found to be the case, for example, by Hill and Hartree in studying the respiration of muscle.

XIV. CHEMISTRY OF CHROMATIN

The chromatin of very few cells has been examined chemically. This is due to the difficulty of obtaining it separate from the other constituents of the cell. Practically only the chromatin of various fish spermatozoa, of some echinoderm spermatozoa, of the corpuscles of geese and hen's blood, of the thymus gland of the calf, have been separately examined. From other cells, however, nucleic acids have been obtained, and these are certainly constituents of chromatin although the chromatin as such has not been isolated. Although our knowledge is hence very scanty, yet the different cells examined have had such different forms and functions and are taken from such different animal species that certain general conclusions may nevertheless be drawn about the constitution of chromatin.

Chromatin apparently consists always of a salt of nucleic acid with a protein base. The nucleic acid is apparently the same, or at any rate closely similar in all the different cells examined; but the protein base appears to be characteristic of the cell. This protein base is either a very basic, simple protein belonging to the group of protamins, as, for example, in the spermatozoa of fish; or it is a histon, that is, a basic simple protein containing more kinds of amino acids in its molecule than the protamins, as for example, the chromatin of birds' corpuscles, the thymus of the calf, or the echinoderm spermatozoa; or it is a more complex and less basic protein of unknown nature in other nuclei. As a matter of fact, it is only in the case of nuclei containing histons and protamins that this protein base has been isolated from the nuclei and its composition determined. In other nuclei protamins and histons do not appear to exist, or if they do exist they are combined with other proteins so that it is impossible to extract them by the simple method of treating with a strong acid, as the protamin or histon is extracted from the cells where it is found.

Kossel (1912) has suggested that there are two kinds of chromatin. In one the protein is in a simple salt union with the nucleic acid, while in the other it is in a firmer union. The former class of nuclei stain readily in basic dyes, the latter less readily.

As regards the inorganic constituents of the chromatin, little can be said. If iron is present, and it does not appear to be so in the head of the fish sperm, or if it is there it is easily extracted with water, it must be in a very loosely combined form. Calcium has been found in fish sperm and echinoderm sperm nuclei. Potassium appears to be absent, judging from microchemical reactions, but entire reliance cannot be placed upon this method for several reasons which have been stated already in my textbook of *Physiological Chemistry*. Recently iron has been found in the nucleoli of nerve and some other cells.

1. *Chemistry of nucleic acid*, $C_{43}H_{57}N_{15}O_{30}P_4$:

For the cytologist this is the most important constituent of protoplasm, for it is this acid which in the nucleus combines with basic dyes and thus gives the nucleus its superior staining ability. It is this which has given the stuff containing nucleic acid the name of the colorable substance, that is, the chromatin. The structure which the cytologist calls a chromosome and in which most see the bearer of all the hereditary traits and some even go so far as to imagine that each trait or character is represented by a distinct unit or gene, this structure as shown in the fixed dyed section is nothing else than a salt of nucleic acid with the basic dye which has been used to stain it. Whether in addition to the dye there is also present some protein matter cannot be definitely stated. Certainly, however, nucleic acid shows the same elective affinity for basic dyes that chromatin shows, so there is no doubt that the capacity for staining depends upon this substance. What, then, is nucleic acid? Are there many nucleic acids, or but one? And what is the function of nucleic acid in the cell? It is alleged by some that the genes in the chromosomes, that is, the protamin nucleate, have the power of self-multiplication. Does the chemistry of these structures throw any light on this possibility?

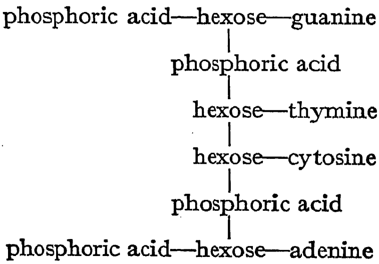
Nucleic acid has been obtained from many different cells and tissues. It is extremely difficult to obtain it in an unchanged condition for the reason that all cells have in them diastases or enzymes which act upon it and immediately after death begin to hydrolyze it. These enzymes are called nucleases.

It is not by any means an impossibility that these diastases are in union with the acid in the nucleus and as soon as respiration stops, that is, as soon as oxidation is reduced, they bring about a decomposition. We may tentatively assume that these same enzymes when there is a supply of oxygen synthesize the nucleic acid. Possibly they constitute some of the protein material usually associated with the chromatin. In line with the general theory sketched at the outset of this paper we may believe as probable that when there is a supply of oxygen coming into the cell, the materials from which the nucleic acid are to be formed are transformed into the anakinetomeres and the enzymes synthesize these forms into nucleic acid, but that as soon as the oxygen supply is withdrawn, these anakinetomeres are no longer produced so that there is a decomposition of that nucleic acid which had already been produced, since the equilibrium is altered. Be that as it may, there is no doubt that a rapid destruction of nucleic acid begins in cells with the death of the cell, and it is necessary, if unchanged nucleic acid is desired, that means be taken to kill or render inactive these enzymes. By plunging the tissues to be examined immediately they are removed from the body into boiling water, strong alcohol, or other reagents which check enzyme action, nucleic acids may be obtained which are more nearly what we believe the acid is in the cell. But the number of typical nucleic acids, that is, nucleic acid containing neither xanthine nor hypoxanthine, but only guanine, adenine, and the pyrimidine bases, are very few.

From the various cells there have so far been two typical acids isolated. The two typical ones are yeast nucleic acid, isolated from brewer's yeast and wheat; and thymus nucleic acid isolated from the thymus gland, many mammalian tissues, and from fish sperm and other cells. The most striking difference between these two forms of acid consists in the difference in the character of the carbohydrate of the molecule. In yeast and wheat nucleic acid this carbohydrate is a pentose, that is, a five-carbon sugar, known as *d*-ribose; in the sperm nucleic acids and thymus nucleic acid, the nature of the carbohydrate is unknown, it is so very unstable, but it is certainly a hexose or six-carbon sugar. There are, however, in some tissues, such, for example, as muscle and the pancreatic gland, and perhaps also in other tissues, not only the hexose containing nucleic acid but also a pentose nucleic acid. Recently it has been maintained by Feulgen and by E. Hammarsten that these two are in some kind of a union. But the evidence for this is not satisfactory. We will come back to it.

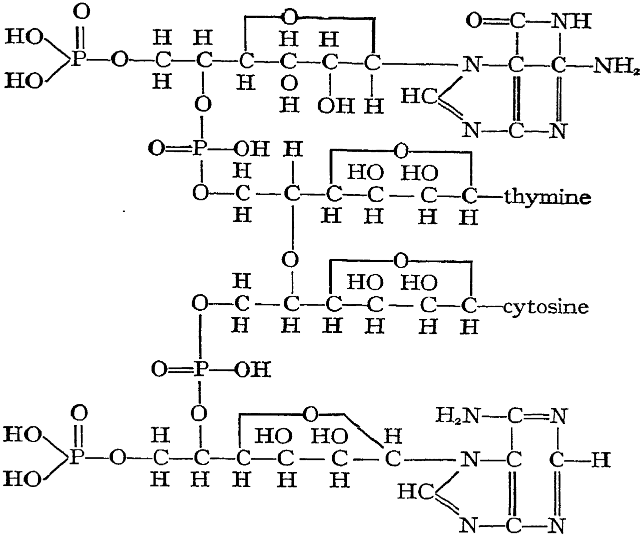
Thymus nucleic acid on decomposition by acid under appropriate conditions yields for each molecule of nucleic acid, four molecules of ortho-phosphoric acid, one molecule of guanine, one of adenine, one of thymine, one of cytosine or uracil; and levulinic and formic acids. The two latter substances show that the molecule contained a hexose sugar, and the quantities as well as other data show that four molecules of such a sugar were present.

By splitting the molecule in various ways so as to obtain different fragments, it has been concluded that it consists of four simpler acids, which are called *nucleotides*, each of these consisting of one molecule of phosphoric acid; one hexose; and a nitrogen-containing base. The following formula has been provisionally assigned to it.



$$\begin{array}{rcl}
\text{phosphoric acid—} & \text{hexose} & \text{—guanine}\\
 & | & \\
 & \text{phosphoric acid} & \\
 & | & \\
 & \text{hexose} & \text{—thymine}\\
 & | & \\
 & \text{hexose} & \text{—cytosine}\\
 & | & \\
 & \text{phosphoric acid} & \\
 & | & \\
\text{phosphoric acid—} & \text{hexose} & \text{—adenine}
\end{array}$$

Nucleic Acid



Thymus Nucleic Acid

The nature of the four carbohydrate groups in thymus nuclei acid is still undetermined. From elementary analysis as well as from the fact that the acid yields levulinic and formic acid when boiled with mineral acids it is believed that hexose is present since hexoses under these conditions yield levulinic and formic acids. It has been impossible to isolate the carbohydrate. If it is a hexose, as seems probable, it is a very unstable form. It cannot be isolated as such. Feulgen, by very cautious acid hydrolysis, has succeeded in obtaining a nucleic acid which lacks guanine and adenine, but which contains the phosphoric acid and the other parts of the molecule. This acid gives a quick reduction with Fehling's solution even in the cold, indicating that in it there are present exposed aldehyde groups. Since the analyses of nucleic acid show always more phosphorus and carbon and less hydrogen and oxygen than the theory requires, he has suggested that the carbohydrate group is glucal, which is an anhydride ketone aldehyde glucose, and which is characterized by its extreme instability in acid. The substitution in the formula of four molecules of glucal in place of those of hexose would give figures for the elementary analyses in better agreement with the actual findings.

The discovery of the very unstable forms of glucose by Irvine (1923), designated by Fischer as γ-glucose, raises the possibility that the hexose may be a γ-hexose. In these γ-hexoses it is believed that some other ring oxide is present than the ordinary form of the butylene ring which exists in ordinary glucose of the α and β form. It has been suggested by Irvine that it is the propylene, or the amylene ring; or it may also be that it is the free aldehyde form itself. The formulas of various kinds of levulose have been given on page 32. At any rate this sugar appears to be in the anakinetomeric form.

The γ sugars are extremely reactive and if they are present in the nucleus in nucleic acid, it is a matter of very great interest. However, all that can at present be affirmed is that there is present a hexose molecule to each phosphoric acid molecule; and that the nature of this hexose is quite unknown, but it certainly is an extremely reactive and unstable form, decomposing as soon as it is set free by hydrolysis from its neighboring carbohydrate, base, and phosphoric acid groups to which it is attached.

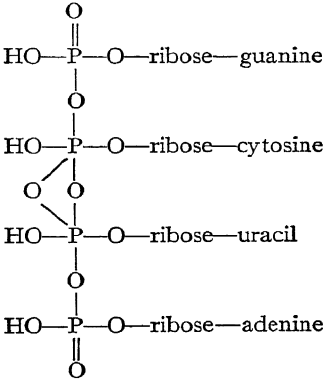
All the complex nucleic acids found in animal cells thus far examined, namely, nucleic acid from the sperm, testis, brain, spleen, liver, intestinal mucosa, pancreas, and other organs, have been found to contain this unstable form of some hexose sugar. Such an acid has not yet been isolated from plant cells, but only two nucleic acids have been obtained from plants and they contain pentoses. It is, however, quite possible that the hexose nucleic acids will be found in plants if these are more carefully examined, just as the pentose nucleic acids have been found in animal tissues as well as in plants.

The recent researches of Feulgen, who has obtained by partial hydrolysis of nucleic acid an acid which reduces Fehling's solution, and which had probably lost only guanine and adenine by hydrolysis, would indicate that these bases are substituted in the terminal aldehyde group. The failure of the acid when intact to reduce shows that the aldehyde groups of its four hexose molecules are all substituted. The study of the individual nucleotides by Levene, Jones, and their associates indicates a similar substitution of thymine and cytosine in the aldehyde group. So it is probable that this group is throughout substituted by these bases. The point of substitution of phosphoric acid in the molecule is uncertain. It is usually placed in carbon atom number 6, at the other end of the chain. This, however, is not yet definitely proved.

Levene suggests that the two pyrimidine nucleotides are united through their carbohydrate groups; and that the terminal nucleotides, the purine nucleotides, are each fastened to the pyrimidine di-nucleotide through phosphoric acid. He bases this on the fact that alkali hydrolysis more readily splits off the purine nucleotides than decomposes the pyrimidine di-nucleotide. But it has not been possible as yet to establish this fact. The linking suggested by Levene would make nucleic acid hexa basic, whereas all observation shows only tetra basicity. There are four sodium atoms in the sodium salt, for example, and not more. The question of the linking in thymus nucleic acid of the different nucleotides is hence uncertain. In yeast nucleic acid, however, it is probable from the ease of alkali hydrolysis that it is through the phosphoric acids, which thus form a polymerized meta-phosphoric acid; on the other hand, Jones believes the union is through the carbohyrate groups of the nucleotides.

There is, however, one other point of interest to the physiologist in Levene's suggestion. It is that the purine nucleotides have each two molecules of orthophosphoric acid substituted in the hexose molecule, thus making a hexose diphosphate. It is of very great interest that such a hexose diphosphate, although of course it may not be this one, is known to be a necessary part of the mechanism of the fermentation of sugar. Hexose diphosphate, as has been shown by Harding, Euler, and others, is an intermediary in this decomposition. It is also maintained, although not yet established, by Meyerhof (1918, 1919) that a similar hexose diphosphate, but one not identical with that in yeast, is the source of the phosphoric and lactic acids (is the lactacidogen so called) in muscle contraction. Whether there is any connection between the presence of this hexose diphosphate in nucleic acid, if indeed it exists there, and alcoholic and other fermentations of sugar cannot be definitely stated, but it is a possibility which derives interest from the suggestion of Gautier that something analogous to alcoholic fermentation, which as is well known takes place in the absence of oxygen, occurs near the nucleus and that the fragments of this fermentation afterward by condensation or by oxidation form the protoplasmic substances.

In yeast nucleic acids the four nucleotides are almost certainly united through the phosphoric acid as Kossel originally suggested. This is shown by the extreme ease of hydrolysis by alkali of this acid as contrasted with thymus nucleic acid. Even dilute alkali if permitted to act for more than a very brief time will hydrolyze off guanylic or adenylic acid or some of the other nucleotides. This acid, therefore, seems to have a backbone of meta-phosphoric acid polymerized as follows:



Yeast Nucleic Acid

Nucleic acid is colloidal in aqueous solution. Its molecular weight according to the formula given would be 1,387. Direct determinations of the molecular weight indicate, however, a polymer of this number. It is probable, therefore, that either by association of two or several molecules, or by direct chemical union of two or more molecules, it exists in larger aggregates. It exists in two forms called respectively α and β. The sodium salt of the α form gels readily; that of the β form, produced by the action of alkali on the α, will not gel. This property of jellying may account in part for the stiff and gelatinous property of the nucleus of many cells.

Free thymus nucleic acid is soluble in water with great difficulty and makes an opalescent solution which undergoes hydrolytic decomposition. It does not coagulate on heating. The individual nucleotides, both the sodium salts (Steudel) and the brucine salts (Levene), have been obtained crystalline; but the tetra-nucleotides have not yet been obtained crystalline.

The barium, calcium, mercuric, lead, platinum, and heavy metal salts are insoluble in water; the sodium, ammonium, and potassium salts are soluble.

In addition to forming salts with inorganic bases of which the calcium salt appears to pre-exist in the fish-sperm nucleus, nucleic acid unites readily with organic bases. Thus it forms salts with all basic dyes, which is the cause of its staining readily with these; it unites with protamin as in the fish-sperm chromatin which is nothing else than the protamin salt; with histon, thus constituting the chromatin of the nuclei of the thymus and birds' red corpuscles; and with other proteins when these are in the cationic or electropositive form due either to their own basicity, as in the case of histon or protamin just quoted, or to an acid reaction in the case of less basic proteins. The fact that it so readily dissolves in ammonia may account for the solvent action of this volatile base on the nucleoli of starfish eggs recorded by the author. These nucleoli are in this case chromatin nucleoli.

It is a fairly strong acid being not precipitated by acetic acid, but only by mineral acids. Being thus a strong acid it is probable that it does not exist as the free acid in cells, but always as the salt of the acid, either sodium, protein, or some other salt. What the mineral bases are which are joined to it in cells is unknown except some calcium, and is badly in need of investigation.

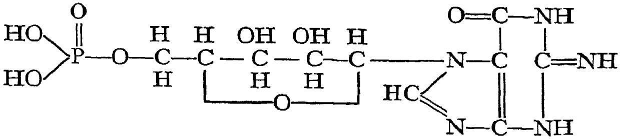
Nucleic acid being thus so readily precipitated by heavy metals and proteins is easily fixed and rendered insoluble in the cell. It is for this reason probably that the chromosomes are usually so well preserved in all kinds of fixatives.

One of the interesting peculiarities of chromatin is that it is very easily soluble in dilute sodium phosphate. As Lynch showed, such a solution is a better solvent than even a more alkaline sodium hydrate solution.

If a solution of free nucleic acid is added to any protein solution it precipitates some of the protein out of solution with it, as a nucleo-protein or nuclein. This is an artificial chromatin and stains like the natural chromatin.

2. *Guanylic acid:*

This is a nucleic acid which yields on hydrolysis only guanine, pentose (*d*-ribose), and ortho-phosphoric acid. Its empirical formula is $C_{10}H_{13}O_8N_5P$. Its structural formula is usually given as:



Guanylic Acid

In other words it is given as a mono-nucleotide. It has been obtained in a crystalline form both as the sodium salt (Steudel) and the brucine salt (Levene). But while it appears thus simple it is in reality colloidal; its sodium salts will gel if sufficiently concentrated, so we are forced to conclude either that it readily polymerizes or that it associates strongly in aqueous solution. It is dextro rotatory. We may provisionally classify it and inosinic and adenylic acids as homo-nucleic acids, the thymus nucleic being a hetero tetra-nucleic acid.

Guanylic acid is obtained from yeast either by partial hydrolysis of yeast nucleic acid, or by hydrolysis with pancreatic extracts. It is also obtained from ox pancreas, which also contains apparently the usual tetra nucleotide, thymus nucleic acid. Both E. Hammarsten and Feulgen believe that guanylic acid exists in the pancreatic gland in union with thymo-nucleic acid, making thus a very complex guanyl-thymo-nucleic acid. It may be obtained from Hammarsten's nucleo-proteid by extraction with boiling water.

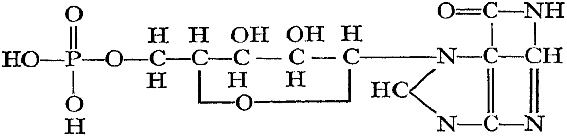
LOCATION IN THE CELLS

The pancreatic gland contains two forms of cells, the duct cells including here the islets of Langerhans, and the acinary tissue proper. It is unknown whether the guanylic acid is found in both or only one of these tissues. Nothing is known of its function or of its location in the cell. If it is attached to thymo-nucleic acid as appears possible but not certain, it is probably in the nucleus. The fact that similar acids (inosinic, guanylic) occur in striated muscle indicates a more extended distribution of this acid than was formerly suspected.

The staining reactions are the same as those of nucleic acid and it forms colloidal solutions. It is precipitated more easily from solution by barium hydroxide than is thymus nucleic acid.

3. *Inosinic acid:*

This is a homo-nucleic acid yielding only phosphoric acid, *d*-ribose and hypoxanthine isolated from mammalian muscle. Its properties are similar to those of guanylic acid. Nothing is known of either its location in the cell, its condition in the cell, or concerning its function. It presumably occurs in the nucleus. It is a dibasic acid.



Inosinic Acid

4. *Basic constituents of chromatin:*

Protamins are strongly basic, simple proteins which thus far have been isolated only from the head of fish sperm. In fact the heads of these sperm consist of a protamin salt of thymus nucleic acid. The protamins are obtained from fish sperm by extracting the latter with dilute sulphuric acid. The protamin goes into solution as protamin sulphate leaving the nucleic acid insoluble. The protamin sulphate thus obtained purified by appropriate methods is found to give the biuret reaction, but most protamins do not give the xanthoproteic, tryptophane, or Millon reaction. They contain usually neither tyrosine, tryptophane, cystine, nor phenylalanine. The free bases are obtained by precipitating the sulphate with $Ba(OH)_2$, are white, non-crystalline, typically colloidal proteins remarkable for great basicity. They are the most basic of proteins, i.e., their solutions in water absorb CO_2 from the air to make carbonates. They are strongly alkaline to litmus, in fact they are stronger bases than ammonia and are not precipitated by ammonia the way histon is. Solutions of protamin either free or as the slightly ammonical protamin sulphate unite with ordinary proteins when added to their solutions to form insoluble salts; the ordinary proteins are less basic, behaving in alkaline solution as acids, the protein being anionic, whereas the protamins remain cationic or electropositive. The salts thus formed between protamin and such proteins as casein, serum albumin, etc., behave like globulins, being insoluble in water but soluble in dilute salt solutions and in dilute solutions of sodium hydrate. In this respect the protamins act like basic lead acetate which precipitates proteins in similar circumstances, or like colloidal ferric hydrate. These protamin proteinates have been very little studied and they would perhaps repay further investigation. It is on the whole unlikely that they exist in cells since were they present protamin-like substances should be extractable from cells, and such substances have not been found elsewhere than in sperm cells. Kossel made the suggestion that each protein molecule had as its nucleus a molecule of protamin, but this is not probable in the light of subsequent work. However, it is possible to isolate tripeptides by prolonged gentle hydrolysis from proteins (Siegfried), and these tripeptides generally contain at least one molecule of arginine, histidine, or lysine, the characteristic components of the protamins.

Protamins have the singular peculiarity of not being digestible by pepsin hydrochloric acid. They are the most resistant proteins known in this regard. This fact makes it probable that pepsin separates the protein molecule into fragments at some bond other than that of a basic and non-basic amino acid. It is not yet known upon what peculiarity of the protamin molecule this resistance depends. On the other hand, protamins are easily hydrolyzed by trypsin and erepsin, being changed first to peptone-like substances known as protones (Kossel, Gato) and thereafter hydrolyzed into their respective amino acids.

It is very interesting that while the nucleic acids of the various sperm chromatins appear to be the same and to consist of thymus nucleic acid, the protamin present seems to be different in each kind of fish, although related fishes such as shad, herring, and white fish have protamins which are closely similar in composition. The protamin of the sturgeon, a ganoid and more primitive type of fish than the teleost, is different from that of the herring. Thus sturin differs from clupein (herring protamin) in that the former contains a large amount of histidine, which is lacking in clupein. Some lysin is present also. The protamin of the carp contains some tyrosine.

5. *Ripening the sperm:*

Accompanying the morphological changes undergone by spermatozoa in their differentiation from spermatocytes to ripe sperm, there is an accompanying chemical differentiation. This chemical change consists in getting rid of what is presumably superfluous. The sperm acquires motility and the nucleus loses nuclear sap, nucleolus, and its surrounding cytoplasm, leaving a sperm head consisting of chromatin. If unripe sperm of the salmon are examined, it will be found that the protamin obtained from them by acid extraction contains not only the basic amino acids, and the small amounts of serine, proline, and valine already mentioned, but a small quantity of many other amino acids. It appears, therefore, that in ripening, the chromatin loses tyrosine, alanine, and all other amino acids than the four already mentioned. It is thus probable that arginine in particular must be of fundamental value, for some reason or other, since it makes so large a proportion of the molecule of this chromatin.

It is of particular interest in this connection that arginine appears to be the only amino acid which is never absent from any protein. It is found in all, although in very varying amounts. Proteins differ enormously in the amount and character of the various amino acids they contain. Thus gelatin lacks tyrosine, lysine, and tryptophane; alanine or glycocoll may be present or absent; histidine, lysine, phenylalanine, are lacking in some; but arginine has been found in all. Proline is also usually present, but is possibly not so important. The fact that the protamin of all the herring tribe of fishes and of the salmonidae, and other forms, consists to so large a proportion of arginine, and the universal presence of this substance in proteins, leads to the belief either that it must be the raw substance from which some very important enzymes are made, or else that it is in some way necessary for the synthesis of the proteins.

The liquor in which the sperm float in the fish testicle has recently been examined by Steudel who has found in it leucine, tyrosine, arginine (in small amounts), agmatine (the base formed from arginine by decarboxylation), and various other amino acids. Evidently the amino acids, split off and discarded as unnecessary, are set free in this liquid. The arginine thus constituting so large a proportion of the sperm chromatin is derived in the salmon from the muscles of the fish. The salmon takes no nourishment from the time he enters the river, and secures his energy from his fat. The ovaries and testes which develop at this time are formed at the expense of the muscle which is undergoing a dissolution. Sufficient muscle is broken down to yield arginine more than sufficient to cover the needs of manufacture of the sperm chromatin. The other amino acids are catabolized, and arginine alone together with small amounts of proline, valine, and serine are saved in the sperm.

The staining reactions of the protamins are peculiar and interesting. They are such strong bases that they will retain their basic character even in fairly strong alkaline solutions, in this respect differing from ordinary proteins in which the acidic character (carboxyl groups) is more developed. Their isoelectric points, in other words, lie well over on the alkaline side. This being the case protamins will unite with acid dyes to form salts, which are often insoluble or very little dissociated, even when the solution is alkaline, under circumstances, in other words, in which ordinary proteins will unite only with basic dyes. Advantage could no doubt be taken of this fact to stain differentially the basic protamins and histons in developing spermatocytes and spermatozoa, and thus follow the formation of the protamins were it desirable. A feebly alkaline solution of some acid dye which precipitates protamin would not stain any ordinary protein, it would not stain nucleic acid or other acid components, and presumably would stain only strongly basic histon or protamin or such inorganic bases as would form insoluble salts with it. By properly adjusting the H-ion concentration in the staining solution, it would be possible to differentiate between proteins of different degrees of basicity, as for example to stain protamin but not histon; or to stain histon and protamin but not ordinary proteins; or to stain histons, protamins, and ordinary proteins, but not stain acid proteins like casein, for example. By means of such methods it should be possible considerably to extend knowledge of the distribution of various kinds of proteins in cells.

6. *Structure of protamins:*

In considering the structure of starch the fact that on digestion with ptyalin maltose is formed indicates that the starch molecule is made up of maltose groups. Just so in the digestion of proteins by prolonged dilute acid hydrolysis, or by tryptic digestion, numerous tripeptide (three amino acid) groups, accompanied to be sure by di-, mono-, and perhaps tetrapeptides make their appearance. This has led to the conception that protamins as well as other proteins may be composed of tripeptide groups. Among the carbohydrates such trisaccharide groups are common. For example, Irvine has suggested that such a group is the foundation of cellulose. Trisaccharides are relatively common whereas no tetrasaccharide is as yet certainly known, although its occurrence would seem equally possible, and our ignorance of carbohydrate chemistry is still so great that no great weight can be placed on negative findings.

That protamin (salmin or clupein) may be composed of tripeptides is strongly indicated by the isolation of such tripeptide groups from them and also by the singular relationship between the number of mono-amino and the basic amino acids. In salmin and clupein as Taylor and Kossel have pointed out there are two molecules of arginine for each molecule of mono-amino acid. There are three of these latter, but there are two molecules of arginine to each molecule of proline or serine. Hence it is suggested that protamin is composed of these groups.

$$\left\{\begin{array}{c}\text{arginine}\\ | \\ \text{valine}\\ | \\ \text{arginine}\end{array}\right\}-\left\{\begin{array}{c}\text{arginine}\\ | \\ \text{serine}\\ | \\ \text{arginine}\end{array}\right\}-\left\{\begin{array}{c}\text{arginine}\\ | \\ \text{proline}\\ | \\ \text{arginine}\end{array}\right\}-\left\{\begin{array}{c}\text{arginine}\\ | \\ \text{proline}\\ | \\ \text{arginine}\end{array}\right\}$$

How many of these go to make up a protamin molecule is uncertain, but if the molecular weight is at least three thousand, it would require for each molecule about eight of these tripeptide groups. The molecule may be more complex than this. A considerable number of protamins of the same percentage composition and of the same amino acid content become possible by changing the arrangement of these amino acid groups. The differences between salmin and clupein may depend on such an isomeric arrangement since chemically these protamins are very similar.

7. *Histons:*

The histons are also basic simple proteins but they differ from the protamins in containing more kinds of amino acids and particularly more mono-amino acids. They thus occupy an intermediate position between protamin and ordinary protein. Instead of the 35 per cent of nitrogen of protamin, the histons contain only about 18 per cent as contrasted with the 13–17 per cent of the usual proteins. They are, hence, less basic than protamins, but more so than ordinary proteins. They contain in other words a larger proportion of basic amino acids than the latter, but far less than the former. Being thus less basic their isoelectric points, while on the alkaline side of the neutral point, are still well removed from that of protamin. They give all the usual protein reactions except that of sulphur as they contain no cysteine or cystine. They give the Millon and also the tryptophane(?) reaction. Like the protamins they are easily but incompletely extracted from cells by dilute sulphuric acid, and they form soluble salts with the usual mineral acids. They form insoluble salts with phosphotungstic acid, phosphomolybdic, tannic, picric, picrolonic, etc. The histons differ from the protamins in that they are precipitated from their solutions by ammonia, particularly in the presence of other ammonium salts; and if added to neutral or feebly alkaline solutions of proteins they will form insoluble salts with the proteins precipitating these from solution. The histons do not coagulate on heating. They contain tyrosine.

The histons have thus far been isolated from the nuclei of the red corpuscles of birds' blood (geese, hens) from the nuclei of cells of the thymus gland of the calf (Kossel) and from pig's testis and lymph glands. In fact the chromatin of birds' red corpuscles and the thymus gland of calves consists apparently of a salt of histon nucleic acid. The histon from birds' blood corpuscles is certainly similar to, but is probably not identical with, the histon from thymus gland. The analysis of the chromatin of birds' corpuscles by Ackerman[1] showed that this chromatin contained apparently no other substances than histon and nucleic acid. He obtained birds' corpuscles by laking and centrifuging the corpuscles. The nuclei remain intact under these circumstances. The hemoglobin dissolves out, and apparently all the other constituents of the cytoplasm if there are any such.

In all these experiments it is always very difficult if not impossible to extract the whole of the histon and thus separate it from the nucleic acid. This led to the possibility that part of the histon might be in some other union than a salt union with the sperm nucleic acid. One part might be dissociable (Kossel proposed to call such nuclei "dissociable" nuclei), the other not. It has recently been shown, however (Steudel and Nakagawa, 1923), that an artificial salt of nucleo-histon acts in just the same way, a considerable part being easily extracted, the other not. Hence no ground exists for supposing that there is anything but a saltlike union of histon and nucleic acid in this chromatin.

8. *The purine bases:*

We have now to ask ourselves why are there purine bases in the chromatin? The carbohydrate is apparently in a very unstable form. We can make some guess, although whether correct or not it is impossible to say, that while it is fairly stable in union with the purines and pyrimidines, just as soon as these are set free it shows its very intense powers of reduction and rapidly oxidizes or burns. Indeed, we are tempted to believe that all the carbohydrates may be first changed to this form in which they are so reactive and in which they can be moved about the cell wherever desired. Just as soon as the purine or pyrimidine is hydrolyzed off from the sugar the latter appears to become very reactive and to undergo auto-oxidation. The union of hexose and guanine, or hexose and adenine, or of the corresponding pentoses appear to be quite stable since these substances, called nucleosides, have been found in various plant cells. But the purines give us a problem which is not yet solved. These substances, however, when partially oxidized have certain peculiarities which may some day be brought into relation with their function in cells. If adenine is hydrolyzed by the enzyme adenase, it loses an amino group in the 6 carbon atom and goes to hypoxanthine. If now it is oxidized by the enzyme xanthine oxidase, it is converted into xanthine and then to uric acid. Now uric acid is very unstable and in alkaline solution undergoes oxidation. In the body of many animals there is also present a diastase (uricase) which converts it directly or indirectly to allantoine. The steps of this process may be as follows:

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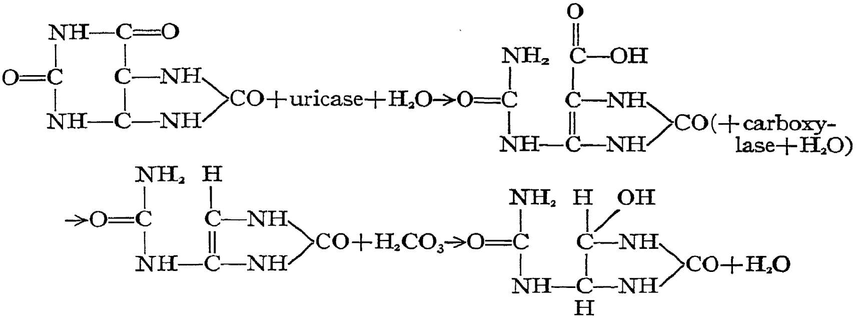
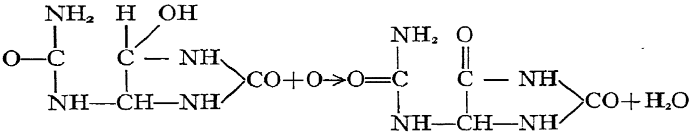
[1] For details see Mathews, *Physiological Chemistry*, 3d ed., pp. 178, 179.

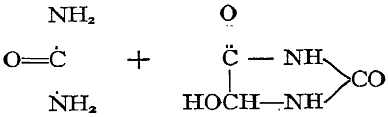


This last substance is auto-oxydizable, one carbon easily losing water and forming bivalent carbon. It will then oxidize itself, particularly if a hydrogen acceptor is present, to form allantoine.



Allantoine

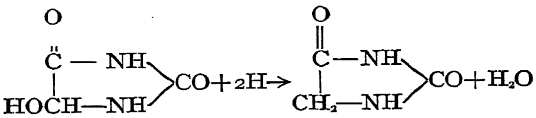
Boiling water will hydrolyze this into urea and allanturic acid.



Urea

Allanturic Acid

Now allanturic acid readily acts as a hydrogen acceptor as follows, hydantoine being formed.



Hydantoine

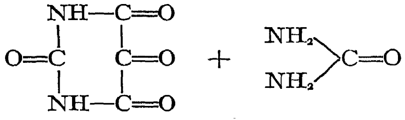
Hydantoine readily hydrolyzes into the carbamide of glycocoll called hydantoic acid, which by further hydrolysis forms glycocoll and carbamic acid; and so ammonia and carbonic acid.

$$\mathrm{NH_2{-}CO{-}NH{-}CH_2{-}COOH} \xrightarrow[+\mathrm{H_2O}]{} \mathrm{NH_2{-}CH_2{-}COOH + NH_2{-}COOH}$$

Hydantoic Acid Glycocoll Carbamic Acid

The essential point of interest in this process besides the light it may throw on the origin and synthesis of purines by the cell is the fact that we have an alternation of reduction and oxidation. Thus the spontaneous oxidation of uric acid to allantoine will probably be facilitated by the fact that the allantoine after hydrolysis to allanturic acid acts the part of a hydrogen acceptor. It thus will accelerate its own formation. Thus allanturic acid will probably be found to hasten the oxidation of uric acid by a reaction by which more allanturic acid is produced. While I do not know that this action of allanturic acid has been observed it should occur. It may thus be taken as a type of what goes on in cells in which such autocatalyses are extremely common. In fact they are the most characteristic things in cells, from a chemical point of view. If the hydantoine thus formed by the allanturic acid oxidizing the uric acid can in its turn revert easily, by taking up oxygen from the air under certain conditions, it would act the part of an oxidase.

But in another way the oxidation of the purines and of the pyrimidines also is very interesting with possibilities. By the oxidation of uric acid by potassium chlorate alloxan and urea are formed as follows:



Alloxan Urea

Alloxan is a very unstable substance and slowly decomposes, probably autocatalytically setting free carbon dioxide. But it is also a very strong acceptor for hydrogen. It is readily reduced yielding dialuric acid. Dialuric acid thus formed has an extraordinary affinity for atmospheric oxygen. It oxidizes itself with the greatest ease back to alloxan. And more than this, it condenses with alloxan to form alloxanthin, which gives a purple salt with barium hydrate and calcium hydrate, but not with sodium hydrate. The interesting thing here is that if uric acid is hydrolyzed in the proper way, it will yield dialuric acid and urea. And the dialuric acid thus set free by hydrolysis is a powerful autooxidizable substance converting itself readily to alloxan, a very reactive compound which again readily takes up hydrogen and returns to dialuric acid. Alloxan acts a rôle such that it should be called a hydrogenase. Alloxanthin also is readily reduced to two molecules of dialuric acid.

This will suffice to show that in the purines and pyrimidines we have substances of a very interesting character which may be playing a very important part in the metabolism of the cell. It is decidedly to be wished that they be examined from this point of view. Certainly dialuric acid is as remarkable from its powers of auto-oxidation as is cysteine. The oxidation goes on also in a neutral solution, just as does that of cysteine; in other words under conditions similar to those in the cell, but this oxidation is not so sensitive to change in H ions as is the cysteine oxidation.

9. *Relation of the chemistry of the nucleus to the theory that the chromosomes are composed of genes; that is, different units which are united in a definite way in the chromosomes and have the power of self-reproduction:*

The foregoing discussion of the composition of the chromatin, while very incomplete owing to the poverty of our knowledge, lends no support to the hypothesis that the chromosomes are made of genes. The best and most convincing proof of this theory which can be had from a chemical point of view is given by the examination of the chromosomes of the fish sperm. Here where there should be the most complex chromatin in the body we find the simplest; namely a salt of protamin and nucleinic acid for which a definite formula may be given. This is the case in the salmon, the herring, and the white fish. It is almost certainly the case also in the sturgeon sperm from which Kossel isolated sturin. Even in the chromatin of the corpuscles of bird's blood a definite salt of histon and nucleic acid has been found; and nothing else. Now, it is very improbable that were the chromosomes constituted of widely different genes they would show so simple and definite a composition. The nucleic acid of widely different cells appears to be the same. Of course it may be different, but the fact that it shows the same physical properties, analysis numbers, rotatory power, and so on indicates that there are probably not several different nucleic acids. Certainly not a vast number. We are, therefore, forced to seek the differences in the protein moiety of the chromatin if such differences exist. Now it is remarkable that nothing of the kind which one would expect on this theory is found in those sperms which we have examined. Always we find but one sort of protamin, containing only three or four different amino acids. While a considerable difference of kind of arrangement is possible even in this one sort of protamin, there is no chemical indication that it exists. The theory of special genes and chromosomal inheritance by unit characters is not supported by such chemical evidence as we have so far obtained. Of course such evidence may be obtained later, but what facts we have point, I believe, to a different explanation of inheritance than this one. But this is not the place to discuss that matter. It can, however, be affirmed that each different kind of cell, so far as it has been examined, does have a different kind of protein in its chromatin. To this extent, at least, the chromosomes of different cells differ. And it is not impossible that differences between the chromosomes of the same cell exist, but it has not yet been possible to put this part of the theory to the test.

Our ignorance of the chemistry of all these complex compounds, however, is still so great that no chemist would be willing to make the affirmation that the chemical facts conclusively disprove the chromosomal theory of inheritance. At the best, or worst, they give little if any support to this view. In the author's opinion, which is here given for what it is worth, the chromatin of spermatozoa is nothing else than the chromatin of spermatozoa; and that of an egg cell is the chromatin of an egg cell. They are not nerve, muscle, epithelial chromatin, in masquerade. While when united they may lead to the formation of all the other chromatins of the body, or at least play an important part in their formation, neither should be regarded as a museum containing samples of all the different products which it is capable of making. For since the number of these products is in fact infinite for each chromatin, as is shown by the differences in cells produced by any change in the conditions of development, by the accidents of existence, such as galls on plants, etc., there is not room in the chromosomes for all these samples. But if the chromatin can make some other chromatins without having a sample to guide it, why not make them all? Why have any samples at all? Considerations of this nature will have different weight with different minds. And it must be remembered that the onus of proof is on those who assert that the chromosomes are such museums containing samples of all the chromatin of all the cells of the body, not only all the chromatins which develop during life, but all that infinite collection of old masters inherited from the past, and all the infinite number of descendants yet to appear in the eons before us, and presenting qualities usually said to be dormant. They are concealed no doubt in the chromosomal attic, ready to be produced when occasion arises.

XV. SUMMARY AND CONCLUSION

In the foregoing section an attempt has been made to bring together in a coherent form modern ideas in regard to energy, the ether, chemical transformations, radiation, and electrochemistry in so far as they bear upon the problem of the nature of life and out of them to construct a theory of the nature of living matter. No attempt has been made to catalogue all the different facts which are known about the chemistry of all the different cells of all kinds of animals and plants. Such an undertaking would require an encyclopedia of many volumes.

We have seen that organisms are, as it were, minute universes moving in a sea of luminiferous ether, and constructed wholly of electrons moving in orbits. The psychic properties such organisms show must in the long run be but the expression of the psychism of the electrons of which they are composed. This ether in its turn is seen to be the mother-substance out of which the electrons are formed; and all that is in them of physical and psychical must be in the ether as well. This ether, it was suggested, is composed of vortices, the etherions, of which the electrons are but two, one of which has received a certain amount of energy from another. The etherion which has received the energy has become the positive electron; and the one which has lost it has become the negative electron. Out of the electrons thus constituted, the whole of the living world has been formed. The ether is itself a great well of energy, but there is as yet no evidence that it exerts an influence of a directive kind upon the electrons, although they are necessarily moving under the influence of its field of force. Organisms appear, as far as our knowledge goes, to be free and independent of control by the ether, although they are composed of minute specks of this substance which have been set apart. The positive electron may be said to be in an anakinetomeric form, since it contains more energy; and the negative may be said to be in the katakinetomeric form.

There are two or three great phenomena of life which have been the subject of this chapter. One is the fact that only partially oxidized protoplasm is irritable. We have considered, then, the chemical basis of the phenomenon of irritability. Another is the fact of respiration. A third is the fact that protoplasm is able to synthesize or to build up, when it is respiring, all the different substances found in it, many of them very complex. A fourth is the fact that electrical currents occur in protoplasm when it is excited or when it is working. No attempt has been made to consider the final fact of the manner in which these syntheses are rendered specific; that is, I have not considered how the syntheses are guided so that certain specific substances are formed and the cell maintains its integrity and increases in size without changing in composition. I have not considered this, because I have had no facts to make a general hypothesis even of a vague kind.

We have now arrived at a general conception of cell life, however, which does correlate the irritable, the respiratory, the synthetic, and the electrical phenomena. The essence of this view is the following: The great difference between living and dead is a difference in the energy content of the molecules and atoms in the two states. All substances, electrons, atoms, and molecules can and do exist in two forms: energy-rich and energy-poor forms. I have proposed to call the former the anakinetomere; the latter the katakinetomere. The former is the living; the latter is the dead form.

The creation of life is the creation of the anakinetomeric form. It is the sun which has created all life, since it is its energy, absorbed by the earth, which has raised the energy content of the molecules and atoms of earth, water, and air until they have become anakinetomeric forms. It is the presence of these energy-rich forms which characterizes protoplasm. This energy is absorbed directly by green plants; and the energy thus absorbed is stored in an immense reservoir for the use of animals, since these are able to utilize but very little of the direct energy of the sun. This great reservoir of energy is the atmosphere, and particularly the oxygen of the atmosphere. The energy of the sun, which has come to the earth in the form of ether pulses, has been absorbed by the oxygen atoms of carbon dioxide in part, and these oxygen atoms have thus been raised to the anakinetomeric form. They are made alive thereby. In one sense at least they are alive. Living matter is living because it has found a method of drawing on the vitality, upon the energy, thus stored in the oxygen.

The oxygen dissolved in water unites with the outer layers of protoplasm. Some of its energy now passes to the molecules of the protoplasm, raising them to the energy-rich, chemically reactive, and unstable forms. For us it is oxygen which thus summons the dead from the tomb; which vitalizes the dead molecules and atoms. The energy is stored in certain of the atoms of the molecules of protoplasm in the form of widened orbits of rotation of the electrons. It is this which gives them the power of reacting and of passing back to the dead. When such electrons fall back to a more stable configuration, the atom and molecule reverts to the dead and inert form such as we keep in bottles. It is the oxygen, then, which vitalizes all animals; but it is from the sun that the vital, radiant energy has come. It is in fact the luminiferous ether which has made things alive, for the ether is the great storehouse of energy; it is itself nothing else than space and time; energy and time. Energy is but ether divided by time. Quantity of energy is quantity of ether per second. So all goes back to the ether; infinity and eternity. From it is derived our energy and life.

Oxygen then united with certain substances in the protoplasm vitalizes; makes them reactive; forms irritable protoplasm. This unstable oxide when it is stimulated passes over the rest of its energy. The energy comes in the form of an electron stream. This causes the electrical phenomena of protoplasm. Some of the protoplasm is at the same time discharged of its energy and passes to the dead or katakinetomeric form. A stimulus then is anything which causes the passage of the energy from oxygen to the protoplasmic constituents; vitalizing some and discharging others. One cannot have the living without the dead.

Thus irritability and electrical phenomena are related. Oxidation, the current of action, and respiration—these are but three names for the same thing. It is seen also that a conductor of the first class is necessary. Such a conductor is furnished by the oriented carbon-chain molecules in the surface film.

An explanation is also at once obtained for all the syntheses of the cell. These are in all cases condensations with dehydration. This is true even in the formation of the carbon chains. It is also true of the condensations to form esters, proteins, glucosides, and all the other materials of protoplasm. They can only occur when the cell is respiring because they occur always between the anakinetomeric, or unstable, energy-rich forms. These forms are created by the energy passed to them from the oxygen.

The processes of oxidation are catalyzed by various diastases or enzymes. Iron, catalase, cysteine, possibly other substances such as oxidases, and substances which act as hydrogenases or reductases play a part. Enzymes or diastases are substances which conduct energy from one molecule to another.

The hydrolytic enzymes take their part in this process, no doubt catalyzing the condensation as well as the hydrolysis. But they can only catalyze in the positive or anabolic direction when they are given the anakinetomeric forms of the molecules upon which to act. Hence they appear to act only hydrolytically. They show their synthetic powers only in the presence of oxygen, which is necessary to form the anakinetomeric forms, and only when the necessary machinery of the cell is present.

Finally a word was said about the composition of chromatin since the fundamental chemistry of this substance is of interest to the student of inheritance. Chemistry as yet gives no support to the gene theory of inheritance, but so far as it touches the problem seems to pronounce against it. Our knowledge is, however, still very fragmentary, and this short account is given to call attention to some of the very fascinating problems which are connected with the chemistry of this substance, rather than to present any definite conclusions.

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SECTION III

PERMEABILITY OF THE CELL TO DIFFUSING SUBSTANCES

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PERMEABILITY OF THE CELL TO DIFFUSING SUBSTANCES

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According to the kinetic theory, molecules, small molecular aggregates, and ions in an essentially liquid medium such as protoplasm, or the various fluids which surround most living cells, have the tendency to become uniformly distributed. This tendency, which is as universal and fundamental as that of heat to flow from a warm to a cool body or electricity from a region of higher to one of lower potential, is utilized by every living cell in securing and distributing materials necessary to its metabolism and in getting rid of waste products. But in no case in a living cell does diffusion proceed in a free and unrestricted manner. Such unrestricted diffusion would soon put an end to that high degree of chemical heterogeneity which is characteristic of all protoplasm and on which life depends. It is a universal property of cells to limit and to modify in a manner which is frequently very complex the diffusion of dissolved substances. The extent of this limitation varies with different cells, with different substances, and with different external and internal conditions, but it is always present, and it is an important task for the physiologist and the cytologist to discover what factors are concerned in determining its characteristic features.

The fundamental nature of the problem of cell permeability—using this term in general to cover the penetration of the cell in either direction by diffusing substances—is at once apparent when the activities of any organism are systematically reviewed. Because of their general interest, a number of examples from human and mammalian physiology may be mentioned in this connection, though such examples might equally well be chosen from a variety of other fields.

Beginning with the alimentary system, the problem of cell permeability arises in many forms. Why, for example, does practically no absorption, even of water, occur in the stomach, while taking place with the greatest ease in the small intestine? Why, in the latter, are some substances absorbed much more rapidly than others; for example, NaCl more rapidly than Na_2SO_4, dextrose more rapidly than sucrose, etc.? Why does NaCl readily enter the blood stream from a solution introduced into the gut but pass with difficulty in the reverse direction? Does the wall of the intestine show evidences of a one-sided permeability to water? What are the means by which water is taken up, not merely from hypotonic, but from isotonic and hypertonic solutions as well? What is the mechanism of normal absorption of the different kinds of digested food materials? How do the various glands connected with the alimentary tract secrete characteristic substances in high concentration? How are the salivary glands able to set up at times in their ducts a hydrostatic pressure higher even than that in the arteries? How is it possible for the stomach to produce hydrochloric acid at a concentration which almost instantly kills most living cells? These questions are merely typical of an indefinite number connected with the alimentary system which in one way or another depend upon cell permeability.

In the case of the circulatory system, similar problems are equally numerous. What is the mechanism by which an exchange of water and dissolved substances is possible between the blood and the tissues? To what extent is lymph formation a process of filtration and to what extent are other factors involved? What differences exist in the permeability of a capillary to various substances, or in the permeability to the same substance of capillaries in different parts of the body (e.g., in the liver, muscles, etc.)? What effect do injuries of various kinds have on the permeability of the capillaries, and how is this effect related to oedema, shock, etc.? How does the body maintain its blood volume at an approximately constant level in spite of the normal and pathological factors which have a tendency to disturb its fluid balance? What is the nature of the exchange of substances between mother and fetus through the placenta? Questions of this sort, too numerous to mention, are constantly arising in physiological and medical work.

The excretory system presents another interesting set of problems involving cell permeability. To what extent does the glomerulus act as a filter? Which substances in the blood does it allow to pass and which does it hold back? What relation do the blood pressure, the osmotic pressure of the blood crystalloids, and the osmotic pressure of the blood colloids have to the rate of urine formation? Why are urea and salts more concentrated in the urine than in the blood, and sugar less so? What is the mechanism by which such concentration is brought about? Why should urea be concentrated fifty or sixty times and NaCl perhaps only twice? How is the kidney able from an almost neutral blood to form an acid urine, and what part does this ability play in maintaining the acid-base balance of the body? To what extent do secretion and reabsorption occur in the tubules? Do all parts of the tubule behave in the same manner? What is the nature of diuresis? Under what conditions do sugar and proteins appear in the urine? Such a list of questions could be extended almost indefinitely.

In the case of respiration, cell permeability is again involved in various ways. The exchange of gases between the lung and the blood and between the blood and the tissues involves the penetration of living cells by gases. Is this penetration a mere case of physical diffusion or does it require a more active intervention of the cells themselves? Can oxygen ever pass from a region of lower to one of higher tension? In the transport of carbon dioxide from the tissues to the lungs and in its elimination in the latter, what is the mechanism by which the reaction of the blood is preserved almost at a fixed point? How are the buffers of the corpuscles and even of the other cells of the body made available for the preservation of the neutrality of the blood plasma? What is the nature of the "chloride shift" from plasma to corpuscles when carbon dioxide is added to the former and the opposite change which occurs when it is removed? It is evident that, like the other activities of the body, respiration involves in various ways the diffusion of substances into and through cells.

In the case of the systems of organs concerned with irritable processes, such as the nervous system, the sense organs, and the muscles, questions of cell permeability might appear to be more remote, except in so far as the cells composing these tissues, like all other cells, receive their nourishment by processes of diffusion. Such, however, is by no means the case. There is much evidence that irritability is intimately connected with changes in cell permeability. Indeed, one of the most important of the theories of irritability is the membrane theory which is discussed at length in Section IV and need, therefore, merely be mentioned here.

Many other examples might be given from mammalian physiology—or equally well from that of any other group of organisms, plant or animal—which would show the fundamental importance of the penetration of the cell by diffusing substances. Indeed, in a certain sense, this question enters into every physiological problem. It merely remains to mention that even in the more purely morphological aspects of cytology such phenomena are also of importance. Apart from their more practical applications in the fixation and staining of cells, they have a certain theoretical significance, since when the microscopist has observed all that is possible of cell structure with the highest powers of the microscope—and perhaps even with the ultramicroscope—he is still able to a certain extent to draw conclusions as to the yet finer structure of the cell from a knowledge of the kind of chemical substances which penetrate it with ease, with difficulty, and not at all. Such conclusions, in the present state of our knowledge, may be little better than useless speculations, but as further information becomes available, it is not at all unlikely that valuable assistance may be obtained in this way in the solution of some of the more difficult problems of cell structure.

In a single section such as the present one it is obviously impossible to review all, or even much, of the work which has been done in as extensive a field as that of cell permeability, especially since this subject is not sharply delimited from numerous other related fields. Even to attempt a complete bibliography would require the use of most of the allotted space. Only certain aspects of the subject will, therefore, be dealt with in this place, and for further information the reader is referred to the larger reviews of Overton (1907), Höber (1922), and Stiles (1921). The topics here chosen for treatment, in the order in which they will be discussed, are: methods of studying cell permeability; the penetrating power of various substances; factors which modify cell permeability; and theories of cell permeability.

I. METHODS OF STUDYING CELL PERMEABILITY

In a general textbook, a detailed account of methods is out of place, but since in the case of cell permeability the results obtained and the conclusions drawn by different workers are so frequently at variance and since in so many cases such disagreement appears to be due to differences in procedure rather than to inaccurate observation, it seems wise before attempting to summarize the present state of our knowledge of the penetration of cells by various substances to give a short description of the chief methods that have been employed in this field in the past, and in particular to point out some of the factors connected with each which are likely to lead to erroneous conclusions. It will appear that no single method by itself is entirely reliable, and the obvious conclusion to be drawn is that in any given investigation as many and as diverse methods as possible ought to be employed before an attempt is made to base generalizations on the results obtained.

Before discussing the defects of particular methods, it may be well to point out certain general sources of error common to all of them. The first is the difficulty of being certain in a given case that the cells employed are in a normal condition. As will be emphasized later (p. 139), a sufficient degree of injury to a cell destroys more or less completely its semi-permeability, and dead cells may readily be penetrated in either direction by most substances. Consequently, it is of the utmost importance that conclusions as to normal physiological cell permeability should be based upon the behavior of uninjured cells only. It is frequently impossible in a given case to be certain that the material in question has been unaffected by the treatment it has received; indeed, with toxic substances such as acids, in other than extremely dilute solutions, it is not possible to study normal penetration at all, since the cell begins to be injured from the outset of the experiment. It is important in every case where injury is at all likely to occur to use all possible means of preventing it, and, if this be impossible, to take this fact into account before drawing any conclusion from the results of the experiment.

A second difficulty arises from the fact that the same method may give very different results with different kinds of material. It is difficult for many workers to see the inherent unlikelihood of finding exactly comparable conditions in all sorts of living cells. Consequently, to take a concrete case, we find one investigator studying the behavior of certain colloidal dyes with plant cells, which, among other peculiarities, are provided with cellulose cell walls, and making the statement as the result of his experiments that the dyes in question do not penetrate living cells, while another worker who has used as his material *Paramoecium*, an animal which not only lacks a cellulose cell wall but which through its food vacuoles is known constantly to ingest large quantities of both liquids and solids, appears to be equally certain that the dyes in question enter living cells generally. The probability is that in the instance cited both workers are correct as far as their own observations go, but wrong in the conclusions drawn from them.

In line with the tendency to generalize excessively from the results obtained with some one kind of cell is the equally common error of attempting from results obtained with some peculiar concentration of a substance and some particular experimental conditions to draw conclusions about the behavior of the substance at other concentrations and under other conditions. For example, a plant cell is placed in M/2 sucrose, and from its osmotic behavior (permanent plasmolysis, etc.) the statement is made that it shows a general impermeability to this substance. Such a statement is certainly not justified without further evidence, for it is possible that (*a*) the strongly hypertonic solution, (*b*) the sugar in high concentration, by some more specific effect, or (*c*) the absence of electrolytes in the solution used may, for the time being, have so altered the cell as to have produced an impermeability which would not be present with a lower concentration or in solutions otherwise more nearly normal physiologically. As a matter of fact, Höber and Memmesheimer (1923) have recently shown that sucrose in relatively high concentrations may apparently decrease the permeability of certain cells. Greater caution should, therefore, be exercised in the future than has been in the past in making the statement, as the result of an experiment, that a cell is generally impermeable to a substance, when all that the experiment in question has shown is that there is an impermeability to some particular, perhaps physiologically, very abnormal concentration of the substance. A somewhat similar case will be mentioned later when it will be shown that the penetration of one electrolyte is greatly affected by the presence of other electrolytes. Consequently, predictions cannot be made from the results of an experiment with pure NaCl, for example, as to the ease with which this substance would penetrate the same cell from a balanced salt solution, or vice versa. In general, in work on cell permeability the complexity of living material must constantly be kept in mind and due account taken of the introduction of every new variable factor.

The methods most used in the past in the study of cell permeability may for convenience be grouped under the five headings: (1) Methods depending on visible changes produced within the cell. (2) Chemical methods. (3) Osmotic methods. (4) Electrical conductivity methods. (5) Physiological methods. Several instances of the use of each method will be given, but in the citation of examples, it has been thought best, for the most part, to select such cases as illustrate the method in its fully developed form rather than to attempt to trace it historically from its first beginnings.

1. *Methods involving visible changes within the cell:*

Most direct of all methods of studying cell permeability are those in which some substance which may be identified by its color is seen under the microscope to enter a cell. The materials which have been most studied by this method are of course the synthetic dyes, though various natural organic pigments and even a few inorganic salts have been used in some cases. Since a special section (p. 135) will be devoted to the penetration of cells by dyes, it is not necessary at this point to do more than to mention as typical examples of the use of the method the papers of Pfeffer (1886), Overton (1900), Höber (1909), and other investigators.

The advantages of this direct method are obvious. It requires no complicated apparatus or time-consuming chemical analyses, and the results, in favorable cases, are unequivocal. If a substance is actually seen to enter a cell, the question of its penetrating power is, with the qualifications just mentioned, definitely settled. However, the method has the disadvantages of very limited applicability, lack of quantitative definiteness (though Collander,. 1921, has attempted with certain plant cells to make it semi-quantitative), and especially, in many cases, of great uncertainty. The fact must constantly be kept in mind that while the coloring of the interior of a cell by a dye proves that the latter has entered the cell, the absence of color does not prove that it has failed to enter. Certain stains such as methylene blue are known to penetrate cells readily and there to become decolorized by chemical reduction or otherwise. Others enter without change, but on account of the thinness of the cell or layer of cells under microscopic observation escape the notice of the observer. Sometimes in such cases the cells may be plasmolyzed with a hypertonic solution, thereby raising the internal concentration of the dye to the point of visibility, but in practice this procedure often fails to give clean-cut results.

The most misleading feature about the use of dyes, however, is the false appearance they give of differences in penetrating power when, as a matter of fact, the observed differences may be chiefly or wholly due to differences in staining ability. Investigators too numerous to mention have fallaciously reasoned, because dye A quickly stains cells and dye B does not, that A enters more readily than B. The actual facts may be the exact reverse. B may enter rapidly, but combining with no constituent of the cell may remain almost invisible, while A, though entering more slowly, may be stored in such quantities as to give an entirely false impression of its penetrating powers. A thoughtful reader of the literature on the permeability of cells to dyes is impressed by the fact that only exceptionally have investigators attempted to distinguish between ability to penetrate and ability to stain, or have even clearly shown that they were aware of the distinction. It has come about, therefore, that what superficially appears to be the simplest and least doubtful method of studying cell permeability has in practice yielded results which perhaps above all others are unreliable and in utter disagreement with one another.

In many cases the penetrating substance itself is invisible, but its presence is made known by visible changes produced within the cell. Thus, alkaloids, caffein, ammonia, etc., form insoluble precipitates with the tannin contained in the cell sap of cells such as those of *Spirogyra*, and this reaction has been used by Overton (1896), Czapek (1910), Tröndle (1920), and others to study the penetrating powers of such substances. Tannin contained in the cell sap may likewise be used to study the penetration of iron salts which give with it a blue color. Szücs (1913) used in this way *Spirogyra* cells and Williams (1918) cells from the leaf stalks of *Saxifraga umbrosa*. The penetration of alkaloids into cells of *Fontinalis* has also been followed by Boresch (1919), who took advantage of the emulsification produced by them of the "Fettknauel" which each cell contains. As long ago as 1875 Darwin observed an increased opacity in the cells of the tentacles of *Drosera* which may have been an indication of the penetration of ammonium carbonate, and Spek (1921) has used somewhat similar reversible changes in the transparency of the protoplasm of *Actinosphaerium* as a criterion of the penetration of a series of salts. As an indication of the passage of salts out of instead of into the cell may be mentioned the observation of Gray (1920) that a precipitation of the globulins of the trout egg occurs when exosmosis of electrolytes has reached a certain point—a change which is readily reversed by the addition of salts to the external medium.

A widely used and valuable method depending on visible changes brought about within the cell by the entrance of substances which are themselves invisible is that involving the change in color of certain intracellular indicators produced by acids and alkalies. These indicators may either be natural pigments of plant cells (Pfeffer, 1877; Haas, 1916*a* and *b;* Brenner, 1918; Jacobs, 1920*a,* etc.) or of animal cells (Harvey, 1914; Crozier, 1916*a, b*, and *c;* 1918*a*) or may be artificial dyes such as neutral red with which the cells have previously been stained *intra vitam* (Bethe, 1909; Warburg, 1910; Harvey, 1911, etc.)

Somewhat different from the examples so far mentioned in being more strictly physiological, but otherwise similar in principle, are such cases as that of the penetration of calcium salts into the root hairs of *Dianthus barbatus* with the formation within the cells of crystals of calcium oxalate (Osterhout, 1909), or of the penetration of sugars, etc., into plant cells with the production within the latter in the dark of starch grains (Boehm, 1883; Meyer, 1886; Ruhland, 1911).

To all of these semi-direct methods the same considerations apply as those already mentioned in the case of dyes, except that such methods have the advantage of a much wider range of applicability—an advantage which is at least partially counterbalanced by the greater likelihood of error due to the more complicated nature of the observed changes.

2. *Chemical methods:*

Less direct and less easy to apply than the method already described, but of much wider application and much better adapted to the securing of quantitative results, is the general method of chemical analysis either of the medium to which the cells are exposed or of the cells themselves. The term chemical analysis is here taken in its broadest sense to include the various procedures employed in physical chemistry such as the determination of freezing-points, of electrical conductivity, hydrogen-ion concentration, etc. The process studied may be either the intake of materials by the cell or their loss (exosmosis) to the surrounding medium.

Examples of the analysis of a medium after cells have been exposed to it are extremely numerous. To mention a few typical cases, Nathansohn (1904*a*) placed disks of *Helianthus* tubers in solutions of various substances, and determined after a time by ordinary chemical methods the amounts of the latter absorbed. Meurer (1909) used in the same way disks of beet and carrot, while Paine (1911) has similarly employed yeast cells, Masing (1912), Kozawa (1914), etc., red blood corpuscles, and Embden and Adler (1922) muscle tissue.

Instead of making ordinary quantitative chemical analyses of the external medium, it is frequently convenient to employ one of the methods of physical chemistry. The conductivity method is especially favorable since measurements may be made by it at frequent intervals without interrupting the experiment or taking away any of the liquid surrounding the cell or tissue. It has been used, among others, by True (1914), True and Bartlett (1915*a* and *b;* 1916) and Brooks (1916*a*) with plant tissues, and by Gray (1921) with fish eggs. The freezing-point method is convenient in some cases and has been employed by Warburg and Wiesel (1912) and others. In cases where the penetration of acids is concerned, hydrogen-ion determinations are sometimes of service (Stiles and Jorgensen, 1915; Loeb, 1922*a*), though if the composition of the external medium is at all complicated the results obtained are difficult to interpret.

The chief advantages of the group of methods just described are their quantitative nature, their applicability to almost any substance and type of cell, and the fact that they permit a series of determinations to be made without any injury to the living material. So great are these advantages that there is some danger of overlooking a number of serious disadvantages. The first is that the amount of substance which disappears from the solution is not necessarily the same as the amount taken up by the cells themselves. If, for example, tissue masses be used, a considerable amount of the material in question may be held in the intercellular spaces, while both with tissues and with separate cells there may be, at times, a concentration at free cell surfaces without actual passage through the latter.

The second difficulty is that the rate of intake of a substance is not necessarily an index of its penetrating power per se. A very important factor in determining the rate of diffusion into a cell is the concentration gradient. If this be steep, movement will be relatively rapid, provided that other conditions are favorable; otherwise it will not be, however permeable the cell may be. It is, of course, obvious that any peculiarity of a cell which brings about the removal of a diffusing substance by metabolic processes, precipitation in an insoluble form, or otherwise will favor the more rapid and more complete loss of the latter from the external solution, giving the appearance of a higher degree of permeability than would otherwise be obtained. It must be remembered, therefore, that there is no necessary exact relation between the rate of disappearance of a substance from the surrounding medium and its true penetrating power.

A third source of error is one which is unavoidable where gross measurements are made which involve a large number of cells, namely, that any dead or injured cells which may be present will contribute to the final result and may give the appearance of a greater degree of permeability than ought to be obtained. Finally, there must constantly be kept in mind the possibility of a partial balancing of the intake of a substance by the loss by exosmosis either of it or of other substances. This condition is especially likely to cause difficulty when the conductivity, freezing-point, or some other non-specific method of measuring concentration is employed, since in such cases no information is furnished about any particular kind of molecule but only about the combined effect of all of those present.

Instead of studying the surrounding medium, the cells themselves may be analyzed. This procedure has the disadvantage of making continuous observations on the same material impossible, since the cells are killed by the process. Also, where analyses are made of tissues, which include substances between, as well as within, the cells, the results do not give an accurate picture of cell permeability. The latter objection does not hold in the case of separate cells, e.g., red blood corpuscles; and information of value may be obtained by the use of such material, especially if simultaneous observations be made on the surrounding fluid (see, for example, Kozawa, 1914, etc.).

One of the most promising methods of applying chemical analysis to cells is that lately used by Wodehouse (1917), Crozier (1919), and Osterhout (1922*a*) in the case of the marine alga, *Valonia*, and by Osterhout (1922*b*), M. M. Brooks (1922), Irwin (1923*a*), and Hoagland and Davis (1923), in the case of the fresh-water alga, *Nitella*. In both of these plants the "cells" are large multicleate structures containing an enormous vacuole filled with sap. From a single *Valonia* "cell" 1, 2, or even 5 c.c. of sap may be obtained; from a *Nitella* "cell" considerably less, but enough for microchemical, spectroscopic, or colorimetric tests. The advantages of the use of this method are that the question of penetration is not in doubt and that the entrance of a given substance into the sap vacuole can be studied quantitatively. Except for the fact that it is uncertain how far conclusions drawn from these rather unusual structures may be applied to cells in general, and that penetration in them is a complicated matter, involving a cellulose cell wall, the entire protoplasmic thickness of the cell, and two free surfaces, the method is an almost ideal one for studying many of the more general aspects of cell permeability.

It is also possible, instead of determining changes in the composition of the medium in contact with cells or tissues, or changes in the composition of the cells themselves, to follow the passage of the material in question through tissues composed of sheets of cells. This is, of course, the general method used in the study of intestinal absorption in mammalian physiology: a question too large for discussion here, but regarding which the reader will find much information and an extensive bibliography in the article by Goldschmidt (1921). For experiments on the general problem of cell permeability, however, the method may be employed in various ways. For example, Overton (1899) used entire tadpoles, the skin serving as the membrane in question. The skin of the adult frog is in many ways admirably suited for experiments of this sort and has been used by Girard (1910), Jacobs (1922), and others. Winterstein (1916*a*) employed in a similar manner the abdominal muscle of the frog, and Krogh (1919*a*), in his work on the diffusion of gases, studied muscle, connective tissue, etc. Among plant tissues, Brooks (1917) employed successfully membranes of *Laminaria*.

The advantages of the membrane method are: first, that large quantities of material may be dealt with, making analysis easy; second, that the medium into which diffusion occurs may be given any desired composition (for example, in experiments with acids, any desired indicator may be used and any amount of buffer materials may be added, the concentration of the diffusing substance may be kept at zero by neutralization, etc.); and third, that this medium may be renewed as often as desired without interrupting the experiment. The disadvantages are that with such large masses of cells it is not easy to distinguish what has diffused through from what has merely diffused out of the cells, and that there is always the possibility of diffusion between rather than through the cells. This latter objection appears frequently not to be as serious as it might seem to be, since results obtained with the membrane method usually agree well with those involving only single cells, but it ought always to be taken into account by means of appropriate experiments in the case of any previously untried tissue.

3. *Osmotic methods:*

Of all of the various methods of studying cell permeability, those depending on osmotic principles have been more used in the past than any others. This has been at least partly due to the fact that the early work in this field was mostly done by botanists, who found such methods especially suitable for use with plant cells. However, even with animal material certain of these methods are both rapid and convenient, requiring no complicated apparatus or troublesome chemical analyses, and have, therefore, in spite of certain obvious defects, found a wide application. The osmotic methods, in general, depend upon volume changes of one sort or another in cells exposed to solutions of the substances whose properties it is desired to test. In the case of plant cells, these changes may be followed readily by reference to the relatively fixed outline of the cell wall. In animal cells, such means of reference are usually not available and recourse must then be had to micrometer measurements in the case of spherical cells such as ova, etc. (Lillie, 1916), the hematokrit method in the case of red blood corpuscles (Kozawa, 1914, 1919), or to weighing in the case of muscles, etc. (Overton, 1902; Meigs, 1912, etc.).

One typical example may be mentioned to show the nature of the method. Overton (1902) placed a gastrocnemius muscle of a frog in a solution containing 0.35 per cent NaCl plus 3.0 per cent ethylene glycol. The osmotic pressure of the solution was equivalent to that of a 2 per cent NaCl solution, i.e., it was strongly hypertonic to the tissues of the frog. The weight of the muscle at the beginning of the experiment was 100 cg.; by the end of fifty minutes it had decreased to 96 cg.; it then increased slowly until at the end of eleven hours it had reached 124 cg. From the fact that the shrinkage produced at first gradually disappeared, Overton concluded that ethylene glycol had entered the muscle, but had done so relatively slowly.

The chief objection to the use of such volume changes as a criterion of penetration is that they may be produced in ways which have nothing to do with permeability. For example, granules of gelatin may swell and shrink in media to which different amounts of acids and salts have been added in a manner which Loeb (1922*b*) has shown is governed entirely by the Donnan equilibrium (see p. 123), the granules being freely permeable to the substances concerned at all times. It is necessary, therefore, in all such work to look carefully for factors other than osmotic ones, such as changes in pH, etc., which might influence the results. Such precautions are especially important in the case of muscle tissue where it is well known that lactic acid is readily produced and that such a production of acid has its own characteristic effect on the volume of the tissue.

With plant tissues, any considerable swelling of the cells is prevented by the cellulose cell walls; the volume changes studied with such material are, therefore, those connected either with the production of, or the recovery from, plasmolysis. The latter process, which may be considered first, has been used qualitatively by so many workers that reference will be made at this point only to the observation of Klebs (1888) that plant cells placed in a solution of glycerol at first undergo plasmolysis and then gradually recover—which he rightly interpreted as evidence of penetration—and to the use of the deplasmolysis method by Osterhout (1911) in his work on the penetration of *Spirogyra* cells by salts.

A very elegant method of following recovery from plasmolysis was first used by DeVries (1884) and later improved by Brooks (1916*b*). The method as employed by the latter was to fasten by one end in a horizontal position under the microscope a piece of the split scape of the dandelion. The apparatus was so arranged that any desired solution could be placed in a vessel surrounding the tissue and movements made by the latter could be observed, greatly magnified, under the microscope. The first effect of a hypertonic solution on a piece of dandelion or other similar tissue so treated is to cause shrinkage of the more elastic cells and therefore to set up a curvature toward the side on which these cells are found. If the substance penetrates, the initial shrinkage gradually disappears, and the process can be followed step by step under the microscope by the straightening of the piece of tissue. Another form of the tissue tension method was employed by Delf (1916) who added the refinement of an optical lever by which movements could be recorded photographically.

Attempts to make the deplasmolysis method quantitative have been made, on the one hand, by Höfler (1918*a*, *b*, and *c*; see also Lepeschkin, 1908*a*) and, on the other, by Fitting (1915). Höfler based his procedure on the following consideration. Suppose a cell whose cell sap has an osmotic pressure P be placed in a large excess of a solution whose osmotic pressure is $2\ P$. If the cell be impermeable to the solute and if complicating factors such as tension of the cell wall, etc., be disregarded, osmotic equilibrium will only be established when the internal pressure has risen to $2\ P$, which, in this case will occur when the volume has decreased to one-half of its original value. Conversely, if in a given experiment it is found that a cell in a certain solution shrinks to one-half of its original size, it may be concluded (always assuming the absence of complicating factors) that the osmotic pressure of the cell at the start of the experiment was one-half of that of the external solution; and similarly that if the shrinkage is to three-fourths of the original volume its initial osmotic pressure was three-fourths of that of the external solution. It follows that when during deplasmolysis the volume recovers from one-half to three-quarters of its original value, the internal concentration at the same time increases by 0.75–0.50 times the external concentration. For example, in one of Höfler's experiments with an external concentration of 0.8M of urea, when the cell had increased from 0.738 to 0.814 of its original volume, calculation would indicate an increase in internal concentration of 0.814–0.738 times 0.8M or 0.061M.

Fitting (1915) has attempted in a somewhat different way to follow quantitatively the process of deplasmolysis. His method is as follows. Pieces of similar plant tissue are placed in a series of solutions of strengths from one slightly below the critical concentration for most of the cells to one which at first plasmolyzes all of them. The disappearance of plasmolysis is then followed simultaneously in all of the solutions. The results of one typical experiment on the penetration of KNO_3 into cells of *Rhoeo* (*Tradescantia*) *discolor* are given in Table I. In each column are indicated the proportions of the cells which showed evidences of plasmolysis.

TABLE I

Length of Time in Minutes	(1) 0.0975M	(2) 0.1000M	(3) 0.1025M	(4) 0.1050M	(5) 0.1075M	(6) 0.1100M	(7) 0.1125M
15.....	few	one-half	three-fourths	many	all	all	all
30.....	none	very few	one-fourth–one-half	three-fourths	many	all	all
60.....	none	none	very few	one-half–three-fourths	three-fourths	many	all
120.....	none	none	none	very few	few	three-fourths	three-fourths

It will be seen that the condition of the tissues in solution (4) at the end of 120 minutes was approximately the same as that of (3) at the end of sixty minutes or of (2) at the end of thirty. It may be concluded, therefore, that during the interval, thirty to sixty minutes, enough of the salt entered to make the cells bear the same relation to a 0.1025M solution that they did at the beginning of this period to a 0.1000M solution, i.e., that the internal concentration had increased from x molecular to $x+0.0025$ molecular. Similarly, there was an increase in the concentration during the following sixty minutes from $x+0.0025$ to $x+0.0050$M. A method very much like that of Fitting has also been used by Tröndle (1918, 1920).

A quantitative method of a different sort, depending not, as does the last method, on changes in the "critical concentration" (i.e., that concentration which is just sufficient to produce plasmolysis) during a considerable time, but rather on the differences in the initial critical concentrations for two different substances, is that of Lepeschkin (1909) and Tröndle (1909). It depends on the fact that there is, in general, a relation between the concentration required to produce plasmolysis and the ease of entrance of a substance. Thus, sucrose enters with difficulty and produces plasmolysis in relatively low concentrations; glycerol enters somewhat more easily and requires a higher concentration; while ethyl alcohol enters very rapidly and does not plasmolyze even in very high concentrations. It is not possible to describe and to criticize the method in question briefly, but it may merely be said that it depends on a comparison of the critical concentration of the substance under investigation and that of another, supposedly non-penetrating, substance such as sucrose. From these two values a numerical "permeability factor" or "permeability coefficient" may be calculated whose value ranges from zero, indicating no penetration, to unity, indicating the most perfect possible penetration. This method has a considerable number of theoretical objections, some of which are discussed by Fitting (1917, 1919) and by Stiles (1921, etc.), and has not been used very much in recent years.

The sources of error in osmotic methods as applied to plant cells are too numerous and too complicated to be discussed in detail here. Mention may merely be made of the following troublesome factors: osmotic changes in the cell during the period of the experiment due to causes other than penetration; exosmosis of osmotically active substances; changes in protoplasmic consistency (see especially Szücs, 1913); injury to the cell from plasmolysis or from the long-continued action of a hypertonic solution; the elasticity and initial tension of the cellulose cell wall which tend at first to obscure the shrinkage of the cell; adhesion of the protoplast to the cell wall, etc. Because of the existence of such factors, it is desirable, especially where it is wished to secure quantitative results, not to depend exclusively on a single one of these methods.

4. *Conductivity methods:*

Determination of the electrical conductivity of cells and tissues and the effect upon it of various external agents have been made by a considerable number of investigators, but the application of the method in an extensive manner to the investigation of cell permeability as such is due to Osterhout, whose book (1922*c*) should be consulted for a full description of his methods and results and for a complete list of his papers bearing on the subject. The living material found most suitable for use with this method is the marine alga, *Laminaria*, which can be cut into disks with a cork borer, the latter stacked like a roll of coins between the electrodes, and the resistance through the pile of disks determined in the usual manner. Although the majority of Osterhout's experiments have been made with *Laminaria*, other plant tissues, including representatives of the green, red, and brown algae, as well as of such flowering plants, and even an animal tissue as frog's skin have been used with essentially the same results.

The question arises: To what extent is conductivity a measure of permeability? It is, of course, certain that the interposition of membranes partly impermeable to ions between two electrodes would greatly increase the electrical resistance of the system, and that the more impermeable the membrane the greater would be the resistance, but in the case of living cells there are other theoretically possible explanations of the high and changeable resistance than the presence of membranes. For example, the electrolytes of the cell might be combined in such a way with other cell constituents as not freely to conduct the electric current, and changes in conductivity might be caused by changes in the extent of this combination. Electrical conductivity is also known to be affected by the viscosity of the medium, the presence of non-electrolytes, etc., and in living cells such factors might easily be imagined to play a part in the production of the observed results.

As to the possible combination of electrolytes, Höber (see p. 118) has shown that at least in one type of cell, the red blood corpuscle, the greater part of the electrolytes are in a free condition; however, it is not necessary to justify the conductivity method by indirect evidence of this sort, since Osterhout has made direct comparisons between the results obtained with it and with other methods, e.g., deplasmolysis (1922*b*), exosmosis (1913), and chemical analysis of the cell sap of *Nitella* (1922*b*), while Brooks (1917) has made a similar comparison with the rate of diffusion through membranes. Since in all of these cases there is a good parallel between conductivity and permeability as shown by the other methods, it may be considered as well established that, for electrolytes at least, the method is reliable. To what extent changes in permeability to non-electrolytes go hand in hand with changes in conductivity is a different question which is in need of further investigation. A further point of some importance in connection with the conductivity method is the extent to which the current is carried between the cells rather than through them. This question has been considered by Osterhout (1921) who has shown, not merely that the greater part of the changes in resistance must be due to changes in the living cells themselves, but has estimated the relative part of the total resistance contributed by the cell walls and found it to be comparatively slight under all conditions.

The advantages of the conductivity method of studying permeability are that it is quantitative, and that, on suitable material, observations may be made continuously over long periods of time with great ease. The disadvantages are that it gives indications only of the behavior of electrolytes and not of that of non-electrolytes, and that while, in the cases that have been well tested, there seems to be a good parallel between conductivity and cell permeability as measured in other ways, there is always the possibility that electrical resistance may be affected by other factors such as changes in concentration of free electrolytes, viscosity, etc. On the whole, however, the method is one which has been proved by experience to be, when used with judgment, of great value.

5. *Physiological methods:*

In addition to the classes of methods already described, there are a number of others of less importance, of which the one most frequently used depends on the effect of dissolved substances on physiological processes of various sorts. Thus, Loeb (1922*a*) has used the cessation of the heart beat of *Fundulus* embryos as a criterion of the penetration of acids and of potassium salts; Overton (1904) the failure of frogs in solutions of potassium salts to show typical potassium paralysis as proof that the salts have not entered the body; Crozier (1918*b*) the withdrawal reaction of the earthworm as an index of the penetration of acids and alkalies; and Jacobs (1912) the difference in the behavior of intracellular structures in *Paramoecium* and in *Vorticella* as evidence of the difference in the ability of carbon dioxide and of other acids to enter cells. To these examples may be added all of the numerous studies which have been made on the action on organisms of drugs and of toxic substances in general. Since a substance usually, though not always, must enter a cell to produce on it its characteristic effects, physiological changes generally indicate penetration; but, on the whole, the physiological method has so many possible sources of error on account of the large number of factors concerned that it must be used with the greatest caution.

II. PENETRATING POWER OF VARIOUS SUBSTANCES

1. *Organic compounds:*

A consideration of the permeability of the cell to various substances may appropriately begin with organic compounds, partly because of their general physiological importance, partly because of the great variety of structure shown by them, which renders them well suited for studying the relation between chemical constitution and penetrating power, and partly because the most extensive, and historically the most important, single piece of work on cell permeability—that of Overton (1895, 1896, 1899, 1900, 1902, etc.)—was concerned chiefly with them. In what follows, except where otherwise mentioned, the data presented are due to this investigator, whose experiments, numbered by the thousands and covering a period of years, included studies on over 500 carbon compounds. For purposes of convenience, the organic acids and dyes, though chemically belonging in this section, will be dealt with more fully elsewhere.

The behavior of the various classes of organic compounds can best be understood if use be made of the conception, unknown at the time of Overton's work, of polar and non-polar compounds (Lewis, 1913, 1916). The former, represented most typically by the inorganic salts and other strong electrolytes, show a type of structure which involves a shifting of one or more electrons from their original positions in the atom in such a way as to lead to the formation either of oppositely charged ions or of a molecule which shows regions of electrical positivity and negativity. The non-polar compounds, on the other hand, of which the hydrocarbons are the most typical representative, have a molecular structure which results from the sharing of electrons by the atoms concerned without the production of regions of great electrical dissimilarity. In general, the polar compounds are highly reactive, ionize readily, have a high dielectric constant and tend to form molecular complexes, while the non-polar compounds are relatively inert, do not ionize, have a low dielectric constant, and have a fairly stable type of structure which can be described as a framework. The polar compounds, on the whole, tend to be more soluble in water (which is itself polar) than in such organic solvents as benzene, xylene, ether, chloroform, etc.; the non-polar compounds show the reverse relation.

In the case of organic molecules, which are frequently large and complex, the molecule may possess portions which are relatively polar and portions which are relatively non-polar. Thus, atomic groups composed of carbon and hydrogen atoms alone, e.g., radicals such as $—C_2H_5$, $—C_4H_9$, $—C_6H_5$, etc., are non-polar in nature; the regions containing such groups as $—COOH$, $—OH$, $—NH_2$, etc., are more or less polar. A compound such as palmitic acid ($C_{15}H_{31}COOH$) thus combines the nature of both classes of substances. The various properties of the molecule as a whole, such as its solubility in different solvents and, most important for the present discussion, its power of entering living cells, seem to depend on the relative predominance of one or the other of these dissimilar types of structure. A number of examples will make this point clear.

The simplest organic compounds in many respects, and the least polar, are the hydrocarbons. These apparently without exception penetrate cells with great ease, equilibrium with the surrounding solution being reached almost instantly. As examples of hydrocarbons studied by him and found to behave in this manner, Overton mentions specifically: methane, pentane, amylene, acetylene, benzene, xylene, naphthalene, and phenanthrene.

Derived from the non-polar hydrocarbons are many compounds containing the more or less polar groups: $—OH$, $—COOH$, $—NH_2$, etc. The behavior of these compounds in the presence of living cells seems to depend partly on the number and character of such groups and partly on the nature of the rest of the molecule. In cases where only one polar group is present, and the remainder of the molecule has the general properties of a hydrocarbon, the latter properties predominate and the compound enters cells without difficulty. Thus, nitroethane, methylamine, and the monohydric alcohols such as methyl and ethyl, alcohols, etc., enter cells readily, the ease of entrance of the alcohols increasing to a certain extent with the increasing length of the carbon chain. The case of the fatty acids, which also naturally belong here, will be dealt with later.

When two polar groups are present in the molecule, the rate of entrance is markedly slowed; and a further increase in the number has an even greater effect. This behavior is very well shown by the alcohols. For example, ethyl alcohol enters cells with great ease, but ethylene glycol reaches an equilibrium with medium-sized cells only after five to fifteen minutes, though this time is shorter with small cells. The trihydric alcohol, glycerol, penetrates still more slowly, producing in plant cells a plasmolysis which usually is fairly slow in disappearing. The tetrahydric alcohol, erythritol, as would be expected, has a rate of penetration slower than that of glycerol, being in fact the highest member of the series whose entrance into cells can readily be shown by the deplasmolysis method. In the case of the hexahydric mannitol this method for the most part yields negative results.

It must not be thought that the greater difficulty of entrance of the higher alcohols is due to the greater length of the carbon chain and higher molecular weight, since the monohydric alcohols corresponding to glycerol, erythritol, and mannitol (i.e., propyl, butyl, and hexyl alcohols, respectively) all enter with the greatest ease, such differences as can be detected being, in fact, in favor of, rather than against, the higher members of the series. Evidently therefore, it is not the size of the molecule but rather the number of —OH groups which determines the difficult penetration of the polyhydric alcohols.

Closely related to the compounds just discussed are the carbohydrates, in which an aldehyde or a ketone group has been introduced into the alcohol molecule. This change appears to make little difference in penetrating power, the various hexoses and the related disaccharides behaving in much the same manner as the hexahydric alcohol, mannitol, as far, at least, as indicated by the plasmolytic method. At this point it should be emphasized, however, that while the experiments of Overton with the method used give useful data about the relative penetrating powers of different substances from hypertonic solutions, their results are apt to be misleading if accepted uncritically. Because Overton could demonstrate in his experiments no penetration of cells by sugars, it by no means follows that such penetration does not occur—and even occur with some readiness. We have, as a matter of fact, the clearest evidence that both in plants and animals the sugars are one of the chief vehicles for carrying into the cell that constant supply of chemical energy on which its activities depend. The same fact has been shown by the direct experiments of Meyer (1886), Ruhland (1911), Brooks (1916*b*), and others. The apparently contradictory results of Overton are to be explained partly by the relative crudeness of the plasmolytic method, and perhaps partly by the fact that a cell, which has been for a time in contact with a hypertonic solution of a non-electrolyte, is by no means unchanged by the treatment. There is some evidence that long-continued plasmolysis may make a cell less permeable, and that a sugar solution, even if not hypertonic, may have the same effect (Höber and Memmesheimer, 1923). It does not follow, therefore, that because a plant cell when plasmolyzed by a hypertonic sugar solution does not regain its original volume, that sugars in small amounts may not fairly readily enter cells. Overton's experiments are to this extent misleading, though his conclusion that, as compared with other substances under the same experimental conditions, sugars penetrate relatively slowly is entirely justified.

Much the same considerations apply in the case of the amino acids. As might have been expected from the presence in these substances of the two strongly polar groups, $—NH_2$ and —COOH, their behavior was found by Overton to be similar to that of the carbohydrates, penetration being exceedingly difficult to demonstrate, though the physiological evidence in favor of their fairly ready entrance into both animal and plant cells is irrefutable. The observation of Höber and Memmesheimer (1923) that amino acids like sugars may diminish the permeability of certain cells may perhaps throw some light on the discrepancy between Overton's results and other known physiological facts.

It has been stated that the penetrating power of organic compounds may be reduced by introducing into the molecule such polar groups as —OH, —COOH, —NH_2 etc. Conversely, the penetrating power may be increased by increasing the non-polar part of the molecule by the introduction of such groups as —CH_3—C_2H_5, and —C_6H_5 in place of hydrogen. It will be noted that this result is produced in spite of the fact that the size of the molecule is thereby increased. The effect is especially striking where the substituted hydrogen atom belongs to a carboxyl, hydroxyl, or amino group. Thus, according to Overton, glycerol and its substitution products, mono-, di-, and triethylin, penetrate with increasing rapidity in the order mentioned. The same is true for the acetic esters, mono-, di-, and triacetin. Likewise, monobutyrin, as would be expected, penetrates more rapidly than monoacetin. The same principle is also well illustrated by the substituted ureas. Urea itself penetrates cells only moderately readily; methyl and ethyl urea more quickly; diethyl urea still more quickly; and triethyl urea almost instantly. Even in the case of the alkaloids, the introduction of an alkyl radical in place of a hydrogen atom may increase the effectiveness of penetration. Thus Overton observed a decided increase in the rate of entrance of cells when a hydroxyl hydrogen atom in morphine is replaced by CH_3, giving the closely related compound codeine. So general does this principle appear to be that Overton even suggested that sugars might by some such substitution be converted into penetrating compounds and so gain entrance to the cell. However, it is not necessary to make such an assumption, since inorganic salts can be shown to penetrate cells in considerable quantities without the possibility of the operation of such a mechanism.

The penetrating power of a carbon compound is also increased when for the polar —OH group a halogen atom is substituted. This is clearly shown in the chlorhydrin derivatives of glycerol. Thus, according to Overton, monochlorhydrin enters more rapidly than glycerol itself, dichlorhydrin still more rapidly, and trichlorhydrin most rapidly of all. This behavior is entirely in accord with that of the halogen derivatives of the hydrocarbons—of which ethyl chloride, ethyl bromide, methyl iodide, ethylene chloride, and chloroform were specifically investigated by Overton—which enter cells with approximately the same readiness as the parent substances.

It appears, therefore, from the facts which have been given, and from numerous others which must be omitted for lack of space, that there is, in the case of organic compounds, a fairly definite relation between penetrating power and chemical structure. In general, a non-polar type of structure seems to favor rapid penetration and a polar type to have the opposite effect. It must not be thought, however, that even the most highly polar compounds are entirely unable to enter cells; that they may do so without any great difficulty will be apparent from the facts cited in the next section. But there is at least a quantitative difference between the two classes of substances, and there are even indications of qualitative differences as well. These differences and certain possible reasons for them will receive attention elsewhere.

2. *Salts:*

As compounds with a high degree of polarity, salts might be expected from the principles found to hold in the case of organic compounds to show a type of penetration which is neither so rapid nor so simple as that of the hydrocarbons and their halogen derivatives. Such is the case; in fact, Overton in his plasmolytic experiments was led to the conclusion that most inorganic salts cannot be demonstrated to enter cells at all, the plasmolysis produced by them being apparently permanent. It is now known that Overton's observations on plasmolysis by salts were probably in error, and his conclusions were certainly too far-reaching; but such experiments at least leave no room for doubt that there is a real and very decided quantitative difference between the rate of penetration of most of the organic compounds mentioned in the preceding section and that of the more typical inorganic salts.

Another line of evidence pointing to a difficulty of penetration of cells by salts or their ions and at the same time indicating a highly complex type of behavior of these substances is found in the comparison of the salt content of certain cells and of the surrounding media. Cases such as those mentioned by Overton (1907, p. 807) where the composition of the ash of several seaweeds is compared with that of the surrounding sea water, are unsatisfactory, since no evidence is given that the salts in the ash are present in a free, diffusible form during the life of the cell; but much more convincing are the figures in Table II from Hoagland and Davis (1923).

TABLE II

ANALYSES OF NITELLA SAP AND OF POND WATER

	Sap (parts per million)	Pond Water (parts per million)	Factor of Concentration
Sodium	230	5	46
Potassium	2,120	*	?
Calcium	410	31	13
Magnesium	430	41	10
Chlorine	3,220	32	100
SO_4	800	31	26
PO_4	350	0.4	870

* No weighable precipitate of potassium chloroplatinate from 200 c.c. of water.

Even in animal cells, which lack a central sap vacuole, there is some evidence of a decidedly unequal distribution of free, diffusible salts. For example, Höber (1910, 1912, 1913), by two independent methods, neither of which, to be sure, is highly accurate, but which give fairly concordant results, has made it probable that at least a considerable portion of the electrolytes in mammalian red blood corpuscles are present in an uncombined form. Nevertheless an analysis by Abderhalden (1898) shows for horse blood the following figures. One thousand parts by weight contain:

	Corpuscles	Plasma
Na	.....	4.358
K	3.326	0.254

It would appear likely, therefore, that the $K^{\bullet}$ ions are prevented from leaving the cell by some other means than chemical combination with cell constituents, while, in the case of the $Na^{\bullet}$ ions, the failure to enter must certainly be due to some other cause, since conductivity, freezing-point, and other studies on blood plasma show that its NaCl is practically all uncombined.

Similar studies on muscle tissue by Höber (1913) yielded results which are indecisive so far as the question of the state of the potassium in the cells is concerned, but as for $Na^{\bullet}$ and Cl', which are almost absent within the muscle cells and are abundant in the blood plasma, the same considerations apply as in the case of the red blood corpuscles. Evidently, in both plant and animal cells, there are factors which prevent the free diffusion of electrolytes to the point where external and internal concentrations are equal. Furthermore, conditions are probably fairly complicated, since Na and K are so closely related in chemical and other properties that it is not easy to imagine any simple mechanism which could be responsible for the highly differential distribution of the two elements.

While considerations such as these make it certain that the diffusion of salts and their ions into and out of cells is by no means free and unrestricted, it is equally certain that cells are not impermeable to them. The salts contained in every cell must at some time have entered it from without, and even a superficial consideration of the known facts of the nutrition of both plants and animals indicates that such an intake must constantly be going on. Indeed, it is an extremely easy matter, by nearly any of the methods of studying cell permeability, to show the entrance of these substances.

For example, to begin with the plasmolytic method, since it was by means of it that Overton had obtained his negative results, Osterhout (1911) showed that it is easy to observe deplasmolysis in *Spirogyra* with solutions of salts of $NH_4^{\bullet}$, $Cs^{\bullet}$, $Rb^{\bullet}$, $Na^{\bullet}$, $K^{\bullet}$, $Li^{\bullet}$, $Mg^{\bullet\bullet}$, $Ca^{\bullet\bullet}$, $Sr^{\bullet\bullet}$, and $Al^{\bullet\bullet\bullet}$ provided that the observations be made continuously. A preliminary plasmolysis (stage 1) is followed under appropriate experimental conditions by deplasmolysis (stage 2); the latter may then be succeeded in turn by a condition called by Osterhout "false plasmolysis" (stage 3) in which the protoplasm appears shrunken away from the cell wall. This condition is an effect of injury and has nothing to do with true osmotic plasmolysis; it may, in fact, be produced even in distilled water. It is evident, therefore, that if observations should be made in a given experiment during stage 1 and then not again until stage 3 had been reached, the conclusion would undoubtedly be drawn by the observer that the plasmolysis was permanent—and according to Osterhout, it was in this way that Overton was led to his erroneous conclusion that salts do not enter plant cells under experimental conditions in appreciable amounts.

The results of Osterhout on this point are by no means isolated; the entrance of a large number of salts has been shown in different cells by osmotic methods of various sorts by Tröndle (1909, 1918), Fitting (1915), Höfler (1918*c*), and others. Examples of the use of chemical methods in obtaining the same result are found in the experiments of Janse (1888), Osterhout (1922*b*), and M. M. Brooks (1922). The production of visible changes within the cell has likewise been found by Osterhout (1909), Spek (1921), etc., to lead to similar conclusions, while, of course, the entire conductivity method depends upon some degree of permeability of the cell to ions, otherwise the current would not pass through it at all. It may be considered, therefore, to be as well established as perhaps any fact in the entire subject of cell permeability that living cells may be entered without serious difficulty by a great variety of salts.

As to the relative ease of penetration of different salts, it appears that there are, as far as the rather scanty available data permit us to judge, considerable, and characteristic, individual differences. These differences seem to be due rather to the nature of the ions of the salts in question than to any other factor—which is not surprising when it is considered that in the aqueous salt solutions which are encountered in physiological work, dissociation, even according to the classical Arrhenius theory is usually at least 80 or 90 per cent complete, while according to the more recent views of Bjerrum, Ghosh, etc., it amounts, in the case of the strong electrolytes, to practically 100 per cent. Salt penetration, therefore, is probably largely, if not wholly, a question of ionic penetration, and the behavior of a given salt must depend on the nature of both of its ions.

It is impossible at present to generalize very far from the small number of quantitative or semi-quantitative observations which have as yet been made on the penetration of cells by salts. As far as the evidence goes, however, it appears that univalent cations belonging to the group of the alkali metals penetrate cells with considerable ease. According to Tröndle (1918) the order for cells from the leaves of *Acer* and *Salix* is: $Li < Na < K < Rb$. Kahho (1921) with the roots of the lupine obtained the order: $Li < Na < K$, as did Spek (1921) also for *Actinosphaerium*.

It may not be out of place to refer at this point to the relatively high degree of cell permeability reported by various workers to ammonium salts. This does not necessarily indicate a particularly high degree of permeability to the ion $NH_4^{\cdot}$, since ammonium salts, especially those with the weaker acids, are hydrolyzed to a considerable extent and their solutions contain in addition to $NH_4^{\cdot}$ ions NH_3 and NH_4OH, one or both of which are known to enter cells with great readiness (see p. 129). The distinction between penetration by an ammonium salt, as such, and by free ammonia in a solution of an ammonium salt is brought out by such studies as those of Overton (1896), Jacobs (1922), Chambers (1922), and Hoagland and Davis (1923).

Of other univalent cations nothing need be said at this point. $H^{\cdot}$ will be discussed elsewhere, while $Ag^{\cdot}$ is so toxic that what has been observed in such studies as have been made upon it is rather its power of injuring than of penetrating cells.

Of bivalent cations, the ones which have been studied most extensively are the physiologically very important alkaline earths. These, in general, enter cells much more slowly than the alkali metals. Thus, Fitting (1915) could find by the method of deplasmolysis no evidence of the entrance of $Ca^{\cdot\cdot}$ and $Ba^{\cdot\cdot}$ and little of that of $Sr^{\cdot\cdot}$. Tröndle (1918) obtained much the same results. But even in the case of these ions there is not complete impermeability. Osterhout (1909), in the experiments already referred to, demonstrated in a highly satisfactory way the entrance of $Ca^{\cdot\cdot}$ into the root hairs of *Dianthus barbatus*, and in a later paper based on the deplasmolysis method reported the entrance of $Ca^{\cdot\cdot}$ and other bivalent cations into plant cells, though stating that it is slower than that of $Na^{\cdot}$. S. C. Brooks (1917) also showed the passage of $CaCl_2$ through a membrane of *Laminaria* tissue, and M. M. Brooks (1922) has recently been able to demonstrate spectroscopically the appearance of Sr in the cell sap of *Nitella* cells placed in a solution of $SrCl_2$. It appears, therefore, that the differences between the alkalies and the alkaline earths are merely quantitative and not qualitative. Of other bivalent and trivalent cations little can be said except that they appear to enter cells relatively slowly except in cases such as those of the heavy metals where the toxicity is great. It is noteworthy that $HgCl_2$, which is only slightly ionized, enters with great rapidity not only living cells but also certain non-living membranes which show a selective permeability, such as those covering the barley grain (Brown, 1909).

In the case of anions, it appears that in general those which are univalent enter cells more readily than those of a higher valence. The familiar differences in mammalian physiology between the behavior in the alimentary tract, the capillaries, and the kidney of NaCl and Na_2SO_4 are probably of this general nature. The order of penetration of anions has been found to be more or less similar in a number of studies involving various types of cells. Thus, Tröndle (1918) found for the penetration rate of Na salts into the palisade cells of certain leaves the order: $SO_4 < Cl < NO_3$, $Cl < Br$, I.

Kahho (1921) likewise found for potassium salts with cells from the root tip of the lupine the order: citrate $< SO_4 <$ tartrate $< Cl < NO_3$, Br. It will be noted that the order of the ions given in both cases is that of the so-called Hofmeister Series; but in the light of the work of Loeb (1922*b*) which shows the predominant importance of the pH in results such as these, the question of the actual specificity of the ions must for the present be left open.

Besides the relatively slow penetration of cells by salts as compared with most organic compounds, there are certain other differences of importance between the two classes of substances. The latter penetrate in a manner which is simple, and their rate of entrance, at least in a few cases which have been investigated, apparently follows Fick's law of diffusion, i.e., the rate, other things being equal, is directly proportional to the external concentration. In the case of salts, however, there is some evidence that this is not the case. Tröndle (1920), for example, obtained the figures given in the second column of Table III, for the rate of penetration of NaCl from solutions of different concentration into palisade cells from the leaves of *Buxus sempervirens*.

TABLE III

RATE OF PENETRATION OF NaCl INTO CELLS FROM LEAVES OF BUXUS SEMPERVIRENS

MINUTES	CONTROL		AFTER TREATMENT WITH 0.005 N HCl	
	Critical Concentration	Intake per Minute	Critical Concentration	Intake per Minute
5	0.9908 M		0.9732 M	
		0.00199		0.00242
20	1.0207 M		1.0095 M	
		0.00232		0.00337
30	1.0439 M		1.0432 M	
		0.00212		0.00419
40	1.0651 M		1.0851 M	

It appears, if Tröndle's figures be trustworthy, that at least in this case, concentration had little effect on the rate of absorption. If, however, the cells had previously been injured, by treatment with an acid such as hydrochloric or oxalic then the rate of entrance of the NaCl became more dependent on the concentration, as the figures in the fourth column show.

In further support of the view that the entrance of salts is a complex process is the fact, according to Tröndle (1920), that it is strongly inhibited by anaesthetics, while that of free alkaloid bases apparently is not—at least to the same extent. It may also be added that the temperature coefficient obtained for various salts by a number of workers is of a much higher order of magnitude than that of ordinary diffusion processes (see p. 149), being, in fact, in many cases of the order which characterizes chemical reactions, while there are some indications that the penetration of organic compounds may have a low temperature coefficient.

To summarize the behavior of salts, it may be said: first, that their rate of entrance is slow as compared with that of the relatively non-polar organic compounds, but that it is sufficiently rapid to be demonstrated by almost any of the methods commonly employed for studying cell permeability; second, that it is highly complicated, in that the equilibrium which tends to be reached is not one of equal concentration inside and outside of the cell, but one in which these concentrations are different—and one in which even with closely related ions such as $Na^{\cdot}$ and $K^{\cdot}$ the ratios of internal to external concentration tend to be very different—and third, that it is readily affected by a variety of conditions such as anaesthetics, injury, etc. So much more complicated is the behavior of the salts than that of the substances considered in the preceding section that Höber (1922) is inclined in the two cases to speak of a physiological and a physical permeability, respectively. That this distinction has much value, however, is doubtful, since it is certainly impossible, on the one hand, to assert that the entrance of alcohol into a living cell is not physiological, or on the other, that the entrance of a salt is in accord with other than physicochemical principles. It is well, however, that the great difference in complexity of the process in the two cases should be recognized and that the more complicated of the two should receive that attention from investigators to which it is justly entitled.

The discussion of the penetration of cells by salts would be incomplete without some mention of a factor whose importance in cell physiology is only beginning, largely through the work of Loeb (1922*b*), to be recognized—namely, the Donnan equilibrium. The earlier workers supposed that an unequal distribution of salts inside and outside of a cell could be explained only by the combination, either chemical or by some vague process of "adsorption" of the salt with some cell constituent or by the prevention of the diffusion of the salt or of its ions by an impermeable membrane. It is now known that there is a third possibility which requires neither chemical nor other combination, nor impermeability of the cell to the salt itself or to its ions, but merely the inability of some other ion or ions—either in the cell or outside it—to pass the cell boundary. Under these conditions, as Donnan (1911) has shown, an unequal distribution of all of the other ions present in the system will be established, no matter how diffusible they of themselves may be.

It may be asked: How far are such unequal distributions of electrolytes as those mentioned on page 118 due to a Donnan equilibrium rather than to the presence of a membrane impermeable to the ions themselves? The subject is too recent to make a definite answer possible in most cases, but at least one instance is now known where this simple physicochemical principle is able to explain facts which had long puzzled physiologists. In the case of red blood corpuscles it had been known for many years that the corpuscles contain less chlorine than the plasma and that the addition of carbon dioxide to the blood causes a shift of chlorine from plasma to corpuscles and vice versa. Among the explanations offered to account for these facts was the improbable one—improbable both from the standpoint of chemistry and of the known facts of cell permeability—that the corpuscles are provided with a membrane impermeable to NaCl but permeable to HCl, and that, on the addition of CO_2 to the blood, HCl was formed in sufficient amounts by decomposition of the NaCl to account for the chloride shift from plasma to cells. It has recently been suggested and partially shown by Warburg (1922) that these facts may be explained by the Donnan principle, and convincing proof of a quantitative nature has recently been given by Van Slyke, Wu, and McLean (1923) that this is the case. In other words, at least a part of the unequal distribution of ions between corpuscles and plasma does not depend on a membrane impermeable to them but on one which is impermeable to some entirely different ion or ions. According to Van Slyke, the results obtained by R. F. Loeb, Atchley, and Palmer (1922) on the distribution of ions between blood plasma and serous cavity fluids can probably be explained in the same way.

However encouraging these applications of the Donnan equilibrium to the complicated question of the distribution of ions between cells and their surroundings may be, it must be remembered that they are still far from furnishing a complete explanation of the known facts. Even in the case of the red blood corpuscles there is the, as yet unexplained, fact that the corpuscles are apparently freely permeable to anions but impermeable to cations: ($H^{\bullet}$ appears to be a possible exception, but the known facts can be accounted for equally well by postulating a permeability to OH' rather than to $H^{\bullet}$). Underlying the Donnan effect, there is, therefore, a more fundamental question of cell permeability of a different sort. Adams (1922) has suggested that the difference in permeability to anions and cations may be accounted for by the nature and magnitude of the electrical charge on the cell membrane, but as yet this theory is unproved.

In the case of other cells, conditions are yet more complicated. The intake and loss of ions in them appears to be a much slower process than in the case of the red blood corpuscles—perhaps indicating the operation of a different factor—and there is no evidence of such a simple equilibrium as in the case described. In fact, figures such as those given by Hoagland and Davis (p. 118) for external and internal concentrations in *Nitella* could certainly not be explained by a simple Donnan equilibrium, for, according to this principle, the ratio of the internal to the external concentration of all univalent cations should be the same and should be equal to the ratio of the external to the internal concentration of all of the univalent anions. How far this is from being the case is apparent from the most superficial inspection of the figures. In the case of the red blood corpuscles, the figures are even more striking, for, from the analyses of Abderhalden (p. 119), the ratio of K inside to K outside in the corpuscles of the horse is about 13:1 while that of Na inside to Na outside approaches zero. It is evident, therefore, from considerations such as these that it is far too early to attempt to explain all of the facts of cell permeability or impermeability to salts on the basis of the Donnan equilibrium in its simplest form; but that the principle is a most important one, which plays a part in all cell processes involving electrolytes, and which will become of greater importance in the future in interpreting questions of cell permeability, is scarcely to be doubted.

3. *Acids and bases:*

The salts which have just been discussed form one group of electrolytes; there remain two others: the acids and bases. These compounds are characterized by a relatively high toxicity due to the very marked degree of physiological activity of $H^{\cdot}$ and OH' ions, respectively. Because the acids have, on the whole, been studied more extensively than the bases they will be considered first and at greater length.

The chief difficulty in the past in the study of cell permeability to acids—and one which perhaps never can be entirely overcome—is that of distinguishing between what may be called primary penetration, the cell in this case remaining relatively normal and the process being analogous to that found in the case of salts, and secondary penetration, where the cell is first markedly injured by the H ions and the acid enters in large quantities only in consequence of this injury. In most of the published work on the permeability of cells to acids no attempt has been made to discriminate between the two factors—with the result that the available data are somewhat confusing and generalizations are hard to make. In this discussion of the subject instead of treating the material historically, an effort will be made, as far as possible, to deal with the two factors separately.

A case involving cell penetration by acids in which the subsequent behavior of the material shows that the degree of injury inflicted on it, if any, must have been very slight is that of the formation of artificial fertilization membranes by means of acids studied by Loeb (1909). The material used in the experiments in question was the egg of the sea urchin, *Strongylocentrotus*, and the method of procedure was to place a quantity of the eggs in a solution (e.g., N/1000) of the acid in isotonic (M/2) NaCl, allow them to remain for varying lengths of time, remove them to normal sea water, and note the percentage which formed membranes. As this general procedure is the first step in Loeb's improved method of artificial parthenogenesis, which frequently yields 100 per cent of normal development, it is probable that such cell penetration as is involved is of the primary rather than the secondary type.

The results of one set of Loeb's experiment are given in Table IV (p. 126). It will be noted with the homologous series of saturated fatty acids, in which each member of the series differs from the preceding one by the addition of CH_2, the effectiveness increases in the order of the size of the molecule, formic acid being the least effective and nonylic the most effective of those studied. This result agrees well with the principle already mentioned (p. 117) that with organic compounds the more highly the non-polar portion of the molecule is developed as compared with the polar portion the more readily primary penetration occurs. Whether this is due to relative lipoid solubility, capillary activity, or some other property of the acid which is similarly correlated with molecular structure need not be discussed at this point; the fact itself is sufficiently clear. The behavior of certain substitution products of the fatty acids likewise agrees with the same principles; for example, butyric acid is more effective than β=oxybutyric acid, propionic more effective than lactic, etc.

TABLE IV

PERCENTAGE OF MEMBRANE FORMATION IN EGGS OF STRONGYLOCENTROTUS

Length of Exposure to N/1000 in Minutes	Formic Acid (Per Cent)	Acetic Acid (Per Cent)	Propionic Acid (Per Cent)	Butyric Acid (Per Cent)	Caprylic Acid (Per Cent)	Nonylic Acid (Per Cent)
1	0	0	0	0	10.0	100.0
1½	0	0	0	.1	80.0	....
2	0	0	.1	10.0	100.0	....
2½	0	.25	20.0	40.0	....	....
3	0	5.0	50.0	90.0	....	....
3½	1.5	60.0	....	95.0	....	....
4	30.0	....	75.0	100.0	....	....
4½	90.0	....	....	....	....	....
5	100.0	....	....	....	....	....

In the same set of experiments, Loeb studied other acids and found that the effectiveness of the mineral acids in causing membrane formation was very slight. He was not able to secure the latter at all with H_2SO_4, while with HCl and HNO_3 the effects were irregular and produced only at fairly high concentrations. In general, his figures support very well the view that in the lower concentrations the mineral acids do not enter in sufficient quantities to be effective, while when the concentration is increased sufficiently to secure adequate penetration, the eggs are killed or seriously injured. In some cases there may be a narrow zone between the region of ineffectiveness and that of serious injury, but this zone with such acids as HCl or HNO_3 is very limited in extent.

Agreeing well with Loeb's results are those obtained by Crozier (1918*b*) on the rapidity with which the earthworm withdraws its posterior end from solutions of various acids. The series obtained by Crozier for the fatty acids was, in the order of effectiveness: formic > valeric > butyric > propionic > acetic. It is seen that this series is the same as that obtained by Loeb with the exception of formic acid, which here shows a relatively greater effect, due doubtless to the fact that it is by far the strongest acid of the series. Results somewhat similar to Loeb's have also been obtained by Philippson and Hannevart (1920) for the action of a number of the fatty acids on muscle; and it has long been known that in affecting the taste receptors of the tongue the fatty acids for a given hydrogen-ion concentration are especially effective (see Crozier 1916*d*).

In a recent paper Brenner (1918) has compared the rate of entrance of a considerable number of acids into the cells of the red cabbage and has tried to distinguish mere penetration, as shown by a change in the color of the intracellular indicator, from injury, as shown by the disappearance of the ability of the cells to undergo deplasmolysis. The general result of his studies has been to indicate that the entrance of the fatty acids is of the primary and that of the mineral acids of the secondary sort. Finally, it may be mentioned that similar results have been obtained by the author in the case of the diffusion of various acids through frog's skin.

It appears likely, therefore, that as far as it is possible to separate primary from secondary penetration the organic acids behave in the same general manner as the other organic compounds discussed on pages 114–18. It is not to be expected, however, that a series of acids with every sort of living material, or at all concentrations, will show any evident correlation between penetrating power and chemical structure. This is partly due to the varying importance of the toxicity factor and partly to the fact that the natural indicators so extensively used in work of this sort are not equally sensitive to strong and to weak acids. The lack of correlation between "penetration time" (as shown by an intracellular indicator) and chemical constitution is apparent in the following figures (from Crozier, 1916*a*, p. 266) obtained by Harvey (1914) with cells from the testis of the holothurian, *Stichopus*, and by Crozier (1916*a*) with certain mantle cells of the mollusk, *Chromodoris*. The concentrations used were in both cases N/100.

TABLE V

CELL PENETRATION FROM 0.01N SOLUTION

Acid	*Chromodoris*	*Stichopus*
	Minutes	Minutes
Valeric (iso-)	1.9	2–4
Salicylic	3.6	0.25
Formic	4.5	2–4
Hydrochloric	7.6	9–11
Nitric	8.4	9–11
Sulphuric	8.5	9–11
Lactic	8.6	9–11
Oxalic	8.8	12–15
Benzoic	9.7	0.25
Monochloracetic	10.0	2–4
Malonic	10.0	30
Tartaric	13.5	30
Malic	14.5	40
Citric	16.0	40
Succinic	16.5	....
Butyric	19.0	45–60
Propionic	30.0	45–60
Acetic	75.0	45–60

It will be seen that with Crozier's material the order is: valeric>butyric>propionic>acetic, but formic acid departs from its previous position probably because of its much greater strength as an acid and consequently greater toxicity than the other members of the same homologous series. With Harvey's material there is little indication of the expected order except that valeric acid is very effective. How far these irregularities are due to the greater importance of the toxicity factor with the material and concentration of acid used by Harvey and how far to the relative ineffectiveness of the weak acids, acetic, propionic, and butyric, in affecting the indicator cannot be decided. That the toxicity factor is an important one at this concentration is shown especially by the fact that in Crozier's work lactic acid is more effective than propionic, oxalic more effective than malonic or succinic, tartaric more effective than malic, etc.—the order in each case being that of the strength of the acids in question and toxicity being usually closely related to strength. Results such as those just described are not necessarily in conflict with those obtained by Loeb with membrane formation, etc.; they merely show the effect of the introduction of the factor of toxicity. Indeed, the relative lack of significance of the order of penetration in concentrations where toxicity is at all appreciable is shown by the fact that Crozier (1916*a*) obtained very different orders for the same series of acids at different concentrations (see the curves on p. 267 of his paper).

It must not be thought that the mineral acids are entirely unable to enter cells without first killing or seriously injuring them. Theoretically, a strongly dissociated acid might be expected to behave much as a strongly dissociated salt, the problem in both cases being largely that of the entrance of certain ions; and it has already been shown that in the case of probably all salts the ions enter cells, though at a relatively slow rate. Consequently, there is no theoretical reason why, for example, HCl should not enter cells slowly in the form of $H^{\cdot}$ and Cl'. There are some indications that mineral acids may enter certain kinds of cells without killing them, but since this penetration in low concentrations is usually slow as compared with that of organic acids, and since in higher concentrations it is complicated by toxicity effects, it is not easy to detect it in most cases.

The subject of the penetration of cells by acids ought not be concluded without reference to the interesting properties of carbonic acid which to a considerable extent depend on those of its anhydride CO_2. Carbonic acid is not only the most universally distributed and important physiologically of all acids, but its behavior with reference to cell penetration is unique. In the first place, molecule for molecule, it appears to enter living cells more rapidly than any other acid. Into starfish eggs stained with neutral red it penetrates so quickly from M/100 solutions that the color change occurs in less time than that required to focus the microscope on an egg placed in such a solution, i.e., in less than five seconds. A comparison by the author, by the frog's skin method, of CO_2 with certain other rapidly penetrating acids also indicates that carbonic acid far surpasses all of the others in the rapidity with which its effects are produced, the only one which at all approaches it being hydrogen sulphide, which is also a gas under ordinary conditions.

A second, and even more important, property of CO_2 is the reversibility of its effects. If starfish eggs stained with neutral red be placed in solutions of various acids, it is relatively difficult even with the fatty acids, and apparently almost impossible with the mineral acids, to produce visible reversible intracellular changes in reaction. Carbon dioxide, on the other hand, may be used almost with impunity to produce such changes. It enters the cell and leaves it with little evidence of causing injury. Similar results may be obtained by the frog's skin method. If small glass tubes containing a solution of a suitable indicator be closed with a piece of fresh frog's skin and placed in solutions of various acids of the concentration of N/100, it will be found that at the time when visible change in the solution, indicating penetration of the acid through the living cells, has occurred the skin is still living and practically normal in the case of CO_2 (as indicated by electrical conductivity and P.D.), entirely dead in the case of HCl, and almost dead in the case of such an acid as butyric.

A third peculiarity of CO_2, which it shares with some other weak and readily penetrating acids such as H_2S, is its ability to produce an intracellular reaction which bears no relation to that of the surrounding solution. For example, the author (Jacobs, 1920*b*) has shown that flowers of *Symphytum peregrinum* may be caused to develop an intracellular acidity in a solution of CO_2 in M/2 $NaHCO_3$, which has the alkaline reaction of pH 7.4, practically as rapidly as in a solution of CO_2 in distilled water, of pH 3.8; i.e., with an apparent hydrogen-ion concentration 4,000 times as great, while in distilled water of pH 5.5–6.0, there is no change, although the apparent H-ion concentration is perhaps 100 times as great as in the first-mentioned solution. Similar observations on other material will be found in other papers by the same author (Jacobs, 1920*a*, 1922*a* and *b*). The relation of this independence of intracellular reaction where CO_2 is one of the substances concerned may perhaps have an important bearing on certain problems of mammalian physiology such as respiration (Jacobs, 1920*b*), circulation (Dale and Evans, 1922), etc.

In principle, the penetration of living cells by bases is similar to that of acids. Strong, highly dissociated bases such as NaOH, KOH, etc., penetrate in appreciable amounts only after injuring the cells, while weaker ones, in whose solutions there are considerable numbers of undissociated molecules, enter much more readily (Bethe, 1909; Warburg, 1910; and especially Harvey, 1911). The following figures from the last-named worker give the times required for N/40 solutions of various alkalies to enter the cells of the leaves of *Elodea:*

	Minutes
NaOH	25
KOH	22
$Ca(OH)_2$	23
$Sr(OH)_2$	15
$Ba(OH)_2$	15
NH_4OH	0.5

A striking instance of the difference between the penetrating powers of NaOH and NH_4OH is found in the following experiment which Harvey describes in the same paper. An *Elodea* leaf stained with neutral red, whose color had changed to yellow owing to the penetration of NH_4OH, was placed in N/50 NaOH, and it was observed that its cells soon become red again just as if they had been exposed to distilled water.

Resembling in many respects the simpler weak bases already mentioned in their penetrating powers are the alkaloids, which Overton and others have shown to enter cells with great readiness, as was indeed to be expected from their known high degree of physiological activity. Further information about the behavior of these substances will be found on page 144.

4. *Water:*

Of all known substances, water is the one which on a priori grounds would be expected to show the most universal ability to enter living cells. Over 80 per cent of the weight and over 99 per cent of the total number of molecules of most active protoplasm are accounted for by this substance, while living cells, with few exceptions, are bathed in an aqueous medium of some sort with which their relation is a most intimate one. The fact that on the average between one and two liters of water enter and leave the human body every day or that, according to Babcock (1912), an annual plant in the production of one pound of dry material requires 200 to 400 pounds of water are indications of the magnitude of the penetration in the aggregate where many cells are concerned.

Nevertheless, the rapidity with which water enters cells is not so great as is often supposed. In plasmolytic experiments with plant cells, the time required to reach equilibrium is frequently as long as an hour or more, while the swelling and shrinking of many animal cells in anisotonic solutions may be surprisingly slow. The very fact that protoplasm in general is not miscible with water would make it seem unlikely that the interchange of this substance through the surface of the cell should be entirely unimpeded. It appears probable, therefore, that while cells in general are permeable to water, the permeability is a somewhat restricted one.

In the case of certain cells, at certain times, the permeability to water is practically zero. Thus, *Fundulus* eggs will develop equally well in distilled water, normal sea water, or sea water concentrated to one-half its original volume, showing during many days no evidence of osmotic effects, provided that the external medium is not physiologically unbalanced with respect to its electrolytes (Loeb, 1912). Facts such as these can be accounted for only by the assumption of an impermeability to water, which, however, may be destroyed under certain conditions (see p. 146).

That a different degree of permeability to water at different times may be observed in the eggs of *Arbacia* has been stated by Lillie (1916, 1917, 1918) who so interpreted the differences in the rate of volume changes of these eggs when placed in anisotonic solutions. Heilbrunn (1920) has objected to Lillie's interpretation, attributing the differences to the changes in the physical state of the protoplasm which are now known to occur after fertilization. It is undoubtedly true that variations in protoplasmic consistency must be taken into account in work of this sort, but admitting such changes, the permeability factor is not thereby entirely eliminated. It must also be remembered that an increase in permeability to salts would not, as Heilbrunn has assumed, necessarily be in conflict with Lillie's findings, since it might be relatively slight and be accompanied by a much greater change in the ease with which water enters.

Instances of one-sided permeability to water have been described in a number of cases, and are of great interest in connection with questions connected with secretion, etc. The mammalian intestine has been so much discussed in this connection and the literature on the subject is so extensive that reference will only be made at this point to the review by Goldschmidt (1921). The skin of the frog is another possibly similar case, though the recorded observations are somewhat conflicting. Overton (1899) originally thought that such a one-sided permeability was proved by the fact that tadpoles shrink in volume when placed in hypertonic solutions but do not swell in ordinary water. He later showed (1904) in adult frogs that the failure of the body to increase in size in water was due to the excretory activity of the kidneys rather than to any impermeability of the skin, and this point has been further investigated by McClure (1919) and by Swingle (1919). The results obtained with isolated skin by various workers (Reid, 1890; Snyder, 1908; Maxwell, 1913; McClendon, 1914, etc.) are somewhat conflicting, but there seems to be little doubt that at least under certain conditions there are evidences of a greater readiness of movement of water in one direction than in the other.

One suggestion as to a possible explanation of one-sided permeability has been made by Bernstein (1905) and by Robertson (1917). It is that the cell membrane is provided with funnel-shaped pores of minute size which may be entered more readily from one side than from the other by molecules in the state of rapid random movement demanded by the kinetic theory. But the impossibility of this explanation is evident from the consideration that if it were possible in this way to set up an increased pressure on the one side of the membrane, then, by means of an osmometer provided with an overflow leading back to the starting-point, perpetual motion could be established.

A much more plausible explanation of such one-sided permeability is furnished by facts connected with anomalous osmosis and electro-endosmosis. It is impossible, in the space available, to discuss the theoretical foundation of this phenomenon, but its general nature will be evident from Figure 1 (p. 132).

Let *AA* represent a section of a membrane penetrated by a pore. It has been found that the walls of such a pore, if it be filled with a solution containing ions, will in general acquire either a positive or a negative charge, the solution at the same time receiving a charge of the opposite sign. This phenomenon has been observed in the case of membranes of the most diverse chemical composition. The origin of the charges may be various; e.g., Loeb (1921) states that in the case of a collodion membrane coated with gelatin it is essentially due to a Donnan equilibrium; in other cases, as with an untreated collodion membrane, it is due in some other way to an unequal distribution of the ions at the phase boundary. If now, in a system of this sort, a difference in electrical potential be set up on the two sides of the membrane, either by means of the passage of a current from without (as indicated in the figure) or by a diffusion potential, etc., in the system itself, the liquid will move toward the side whose charge is opposite to its own. If concentration differences exist on the two sides of the membrane, this electrical flow of liquid may assist or may act in opposition to the osmotic flow, as the case may be, leading in some instances to the apparently anomalous condition of a flow of water from a region of higher to one of lower osmotic pressure.

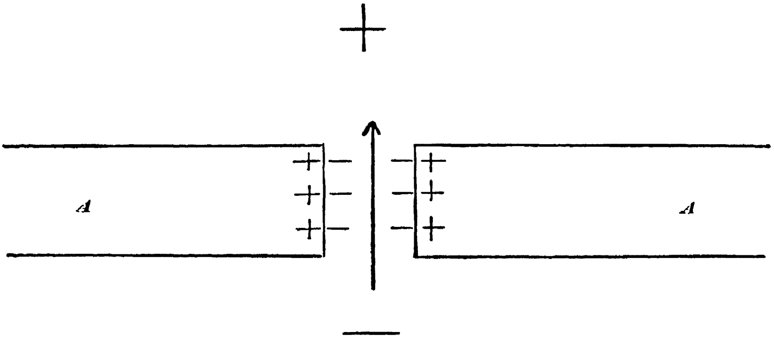

FIG. 1

The case represented in the diagram is only one of a number of possible ones; i.e., the membrane may be positively or negatively charged, or uncharged, and the external potential difference may be in the direction indicated, in the reverse direction, or lacking. These conditions are represented diagrammatically in the paper by Bartell and Madison (1921) who in the same paper give data showing that in a considerable number of cases, involving different electrolytes and different membranes, the flow is what the theory demands. Loeb (1922*c*) has also shown that the rate of transport of water through collodion membranes may be accounted for semi-quantitatively by the observed potential differences.

The possible application of facts such as these to problems of cell physiology are obvious. That electrical potential differences on the opposite sides of membranes such as frog's skin, intestine, etc., exist, has been known for many years, and there are good reasons for believing that there may be similar differences in the case of ordinary cell membranes. It is also known that in the presence of proteins, which enter so largely into the chemical composition of the cell, conditions would be especially favorable for the establishment of electrical charges on the walls of the hypothetical pores, these charges being easily influenced through the Donnan equilibrium by changes in the pH of the medium, etc. There is no difficulty, therefore, in constructing an imaginary picture of a possible mechanism for producing in cells a one-sided permeability to liquids, a flow of water from a region of higher to one of lower osmotic pressure, etc. However, in the present state of our knowledge such a picture, while highly suggestive, must be regarded as purely speculative.

5. *Gases:*

That at least certain gases may penetrate living cells with great readiness is indicated by a number of well-known facts such as the general and constant intake of oxygen and elimination of carbon dioxide by all aerobic cells, and the reverse process in green plants during photosynthesis. The mammalian lung is also freely permeable to many gases; not merely to oxygen and carbon dioxide but to nitrogen, carbon monoxide, toxic war gases, etc. The cells of the alveolar epithelium are, to be sure, so highly specialized that general conclusions cannot be drawn from their behavior, but other cases in the mammalian body illustrate the same principle. Thus, air or another gas mixture introduced into a closed cavity such as the mouth (Henderson and Stehle, 1919), stomach (Edkins, 1921), abdomen (Haggard and Henderson, 1919), or even subcutaneously (Campbell, 1923) changes fairly rapidly in composition in such a manner that each of the gases normally present in the body tends to approach a definite tension, which presumably is that of the surrounding tissues. So great is the power of gases to pass through tissues into the circulation that Haggard and Henderson (1919) kept an animal alive for several hours solely by means of pure oxygen introduced into its abdominal cavity.

Facts such as these indicate that the diffusion of gases into living cells is a very general phenomenon; there are, however, as yet very few quantitative data which enable exact comparisons to be made between the penetrating powers of different gases into the same tissue or of the same gas into different tissues. A beginning in this direction has recently been made by Krogh (1919*a*) who used in his experiments an apparatus consisting essentially of two chambers separated by a thin sheet of known thickness of such a tissue as muscle or connective tissue. The gas under investigation was allowed to diffuse from a definite tension in the first chamber into the second chamber where its tension could be kept practically at zero by the use of a suitable absorbent. From the amounts of gas penetrating the partition in a given time, Krogh was able to determine for oxygen, carbon dioxide, and carbon monoxide the constants given in the first column of Table VI, which are proportional to the relative rates of diffusion of these gases through several animal tissues under comparable conditions. In the second and third columns will be found similar figures obtained by Hüfner and by Exner for water and for soap films, respectively. The fourth column gives the corresponding figures calculated on the simple physical assumptions that the diffusibility of a gas into water or through a water-soaked film is directly proportional to its solubility in water and inversely proportional to the square root of its molecular weight. It is seen that the agreement in the three observed cases with the theoretical rates is, on the whole, good. The figure for carbon dioxide with living tissues is somewhat high, but Krogh states that it was based on one experiment only; it is also possible that certain other factors may play a part in this case. The general conclusion to be drawn from these experiments, which, to be sure, are not very extensive, is that the diffusion of at least certain gases into living cells is a relatively simple process.

TABLE VI

Diffusion Rates of Gases

	Krogh (Animal Tissues)	Hüfner (Water)	Exner (Soap Solution)	Calculated (Water)
Oxygen	1.0	1.00	1.00	1.00
Nitrogen	0.4–0.7	0.53	0.44	0.53
Carbon monoxide	0.73	....	....	0.89
Carbon dioxide	35.70	27.50	24.20	23.10

The question of changes in the permeability of individual cells to gases has been much discussed as a possible mechanism for regulating the intensity of metabolism. Thus, it has at different times been suggested that the increased metabolism which, for example, follows fertilization may be due to an increased permeability of the egg to oxygen; or that a change in permeability to carbon dioxide may have a similar effect, the retention of this substance slowing the chemical processes which lead to its production (Lillie, 1909). In the case of oxygen, Lyon and Shackell (1910) have attempted to obtain evidence of changes in permeability in the following manner: *Toxopneustes* eggs, fertilized and unfertilized, were stained with methylene blue, then decolorized in a stream of hydrogen in an Engelmann gas chamber. On the re-admission of oxygen, the color returned at an equal rate in the two lots of eggs, which the authors interpreted as indicating no difference in the rate of penetration in the two cases. Harvey (1922) also compared the behavior of living and killed tissues of various sorts when treated in somewhat the same manner, and found no significant differences in the two cases. He likewise concluded that an increased rate of penetration of oxygen is not an important factor in stimulation.

As to carbon dioxide, the author has at various times stained starfish eggs with neutral red and attempted to detect a difference in the rate of penetration in fertilized and unfertilized eggs, but this is so rapid in both cases that no certain differences could be observed, the color of the indicator changing in less than five seconds in all of the eggs. While negative results such as these do not prove that there is no increased permeability to gases following fertilization, it may be pointed out that an increase in permeability to water and water-soluble substances such as has been described by Lillie (1916*b*) and others would not necessarily be expected to be correlated with an increased readiness of penetration of gases, since oxygen and carbon dioxide are, as a matter of fact, less soluble in water than in fatty substances (Vernon, 1907).

6. *Dyes:*

The consideration of dyes in a section by themselves is purely a matter of convenience, since chemically they do not form a group which is either homogeneous or sharply separated from colorless substances. But, practically, in a work such as this, it is desirable to treat them as a class, partly because of their great importance in cytological work and partly because of the rôle they have played in the past and are likely to play in the future in connection with the theoretical aspects of the general question of cell permeability.

In many respects the dyes appear to be a group of substances offering unique advantages for the testing of the various hypotheses which have been advanced in this field. Their penetration may be observed easily and directly; they may be used with a great many kinds of living material of both plant and animal origin; and, best of all for the testing of theories, dyes may be secured which give almost any desired combination of chemical and physical properties, such as acid or basic nature, varying molecular weight, colloidal or non-colloidal behavior in aqueous solutions, high or low solubility in lipoids, etc. However, in spite of these advantages, the results obtained with them have on the whole been disappointing. They have not only not as yet furnished a solution of the general problem of cell permeability but they have, if anything, been responsible for even greater confusion than existed before.

The reasons are not hard to understand. The very ease with which staining methods can be employed has tempted into the rather difficult field of cell-permeability workers who would have been discouraged by the necessity of making, for example, accurate chemical analyses or determinations of electrical conductivity—with the result that amateurish work on this subject is only too frequent. The diversity of living material available has also to a certain extent been a cause of confusion because of the difference in the results obtained with different kinds of cells and the failure of many investigators to make allowance for these differences in framing their hypotheses. In addition, there has been, as has already been mentioned on page 104, a general failure to distinguish between the staining power of a dye and its penetrating power, though these two properties bear no necessary relation to each other. For these and certain other reasons, the literature on the subject is in a highly unsatisfactory state. Not only is it impossible from the mass of conflicting data to give any general explanation of the behavior of dyes, but it is impossible even to be certain of many of the facts on which such an explanation must ultimately rest. Since it is not desirable here to go deeply into controversial matters, there will be given merely a brief semi-historical account of some of the work which has been done with such comments as appear to be justified.

Among the earliest workers on the penetration of cells by dyes may be mentioned Pfeffer (1886) and Ehrlich (1887), the former, working on plant and the latter on animal cells, especially those from the nervous system. Pfeffer studied in all about twenty dyes, and found that some penetrate living cells and others do not, the former, for the most part belonging to the class of the basic dyes. Ehrlich obtained similar results, and also made the important generalization that dyes which are "neurotropic," i.e., which have an affinity for nervous tissues, are also "lipotropic," i.e., have an affinity for fatty materials.

Some years later Overton (1900) made an extensive study of cell penetration by dyes, using about forty of the latter and employing as his living material various plant and animal cells. He had already been led by his studies on the penetration of cells by colorless organic compounds to the conclusion that the lipoid solubility of a substance is that factor which more than any other determines its ease of entrance, and his studies on dyes seemed to confirm this view in a satisfactory manner. Of the stains employed, those which entered cells were also taken up readily by cholesterol, lecithin, protagon, and cerebrin, and those which did not enter were not. Overton concluded, therefore, that these experiments supported in a satisfactory manner his lipoid theory.

The work of Overton was not long allowed to pass unchallenged. Beginning in 1908, Ruhland published a series of papers (1908*a* and *b*, 1912, 1913*a* and *b*, etc.) in which he showed: first, that there are dyes which are not soluble in lipoids and nevertheless stain plant cells readily (e.g., methylene green) and second, that there are lipoid soluble dyes which enter with great difficulty (e.g., rhodamin). He was led by these observations to reject the Overton theory *in toto*, and he suggested as an alternative theory at first that the important factor in determining penetration is the basic or acid nature of the dye and later (1912) that it is the size of the molecule (or molecular aggregate), colloidal dyes not entering cells while non-colloidal ones do, and the ease of entrance being correlated with the size of the molecule. Küster (1912), who also worked with plant cells, came to much the same conclusion. About the same time Garmus (1912) pointed out similar exceptions to the Overton rule. He found that lipoid-soluble dyes such as pheno-safranin, rose bengale, etc., failed to stain the cells of certain glands in the nictitating membrane of the frog, while lipoid-insoluble dyes such as thionin were able to stain them, and in consequence he also rejected the lipoid theory.

Somewhat earlier Höber (1909) had also found that the lipoid-soluble Echtrot A, cyanosin, etc., do not stain *Spirogyra*, while the lipoid-insoluble methylene green, thionin, etc., do. But he has been more cautious than the other workers mentioned in entirely rejecting the Overton view. In his latest excellent discussion of the whole subject (1922) he admits the theoretical importance of exceptions such as those mentioned, but points out that most of the cases in which lipoid-soluble dyes apparently fail to enter cells are those where colloidal dyes and plant cells are concerned and where perhaps the cellulose cell wall may be a complicating factor—at any rate, the same dyes very frequently stain animal cells. Exceptions of this sort to the Overton principle are, therefore, extremely rare. As to the undoubted entrance of cells by lipoid-insoluble dyes, he considers it to be analogous to the entrance of salts, involving a physiological, as opposed to a physical, permeability. While this terminology is perhaps unfortunate, there is no theoretical objection to the view that the processes involved in the two cases may be of a different nature. In support of this view, Höber mentions the observation of Hertz (1922) that the entrance of lipoid-insoluble dyes into *Opalina* is hindered by narcosis, while that of lipoid-soluble dyes is not. In further support of this view may also perhaps be the fact that whereas Pfeffer (1886) found that the effect of temperature on the staining of plant cells by the lipoid-soluble methylene blue was slight, as would be expected for a process of simple diffusion, Collander (1921) obtained for lipoid-insoluble acid dyes a temperature coefficient of the order of magnitude of that belonging to chemical reactions. Collander likewise reports a decided effect of anaesthetics on the staining of cells by the same class of dyes.

The general conclusion arrived at by Höber is that Overton was wrong in denying the entrance of all lipoid-insoluble dyes just as he was wrong in denying the entrance (under experimental conditions) of salts. His lipoid theory, therefore, as a full and complete explanation of the behavior of all substances, is certainly inadequate. But that lipoid-solubility is a property of a compound which favors a ready entrance into living cells seems on the whole to be borne out by experiments with dyes. When it is remembered that the absence of visible staining does not necessarily prove failure to penetrate and that, further, most of the exceptions to the principle that lipoid-soluble dyes enter cells have been reported in the case of the combination of circumstances presented by a colloidal dye in the presence of a plant cell possessing a cellulose cell wall, and do not apply to at least certain animal cells, it seems advisable to retain for the present this part of the theory as a working hypothesis.

Within the last few years two workers, Nierenstein (1920) and Collander (1921), respectively, have given a certain amount of support to the first portion of Overton's position. Nierenstein, using *Paramoecium*, studied the staining power of over 100 dyes, and compared it with the partition coefficients of these dyes between: (1) olive oil and water, (2) olive oil plus oleic acid and water, (3) olive oil plus diamylamine and water, and (4) olive oil plus oleic acid plus diamylamine and water. The correlation between staining power and the first partition coefficient was poor, between staining power and the second and third was good for basic and acid dyes, respectively, but poor for the two classes of dyes in the reverse order; between staining power and the fourth was good for all of the dyes used without exception. It will be noted that these results while supporting, in this modified form, the Overton theory, perhaps have to do rather with the question of the staining of cells than of their penetration. Nierenstein's conclusions have been criticized by Collander (1921), but since the two workers used such different material in their experiments it is to be expected that their results would be different.

Collander's work was done with acid dyes, and his results are supposed to support the contention that the relative ineffectiveness of these dyes in staining cells is not due merely to failure to combine with cell constituents as Ruhland (1913*b*), Bethe (1922), and others have supposed, but to actual failure to enter the cell in more than minute amounts. His evidence is based on a method of estimating the concentration of the dye within the cell and the use of the method to show that the internal concentration after many hours may be no more than 1/8 to 1/160 of the external concentration. Unfortunately, in most cases, he does not rule out the possibility that this difference is a partition effect rather than one due to difficult penetration. In the latter case, it should be easy to show that the concentration of the dye gradually increases from hour to hour or from day to day until it approaches or equals that in the external liquid; in the former case the low value presumably represents a condition of equilibrium which ought not to change. In some cases, evidence is given of a continuous slow penetration but, for the most part, the figures are merely those obtained from a single observation at the end of a day or so, and hence are inconclusive. Collander in his final discussion is, on the whole, rather favorably inclined toward the first portion of the Overton theory.

Before leaving the subject of cell penetration by dyes one additional point of view must be mentioned. Bethe (1922) and Rohde (1917, 1920) have studied the effect of the reaction of the medium and of the cell on the staining power of various dyes. Bethe, after pointing out the fact, already well known, and giving further experimental proof of it, that proteins in sufficiently alkaline solutions combine with basic but not with acid dyes and in sufficiently acid solutions show the reverse behavior, expresses the view that the staining of living cells more readily by basic than by acid dyes is due, not to any difference in the rates of penetration of these substances, but rather to the fact that the reaction of most cells is such as to make possible a combination of the cell proteins only with the former. In the case of cells having a sufficiently acid reaction, staining with acid dyes should occur more readily than with basic ones, and this both Bethe and Rohde believe they have shown to be true in the blood corpuscles of ascidians, in such plant cells as those of unripe apples, etc., and in animal cells artificially made acid in reaction. The latter result Rohde (1920) claims to have brought about on the frog by feeding the animal boric acid until the pH of the blood had been reduced to 4.2 (!). If this surprising figure was correctly determined, it is questionable whether the cells of the body were sufficiently normal to permit conclusions to be drawn; but, at any rate, Rohde states that, under these conditions, acid dyes show a marked contrast to their usual behavior, staining cells, being absorbed from the intestine and being excreted by the kidney more freely than basic ones, while the latter, in animals made alkaline by the feeding of Na_2CO_3, showed an even greater activity than normally.

Rohde's results have been criticized by Collander (1921), who states that it is not generally true that acid dyes stain plant cells with an acid reaction more readily than those whose reaction is less acid, since in his experiments the plant cells which took up these dyes most strongly were not characterized by a high degree of acidity. It seems impossible at present to decide this point definitely. It may be mentioned, however, that while the factor mentioned by Bethe and Rohde must undoubtedly be of importance in the staining of cells, its importance in their penetration, as such, is perhaps somewhat less certain. On page 144 will be mentioned several further considerations which must be taken into account in work of this sort.

III. FACTORS WHICH MODIFY CELL PERMEABILITY

1. *Injury and death:*

As familiar examples of the effects of death on the permeability of cells may be mentioned the escape of the red pigment, salts, and sugar when a piece of red beet is placed in boiling water, and the penetration by stains and other cytological reagents of tissues after "fixation." Facts such as these have been known for many years, but the unsatisfactory nature of purely qualitative information has recently led a number of workers to attempt to study in a quantitative manner the changes in permeability which occur with death. By far the most successful of these attempts has been that of Osterhout (1922*c*), who has used his conductivity method as a convenient and at the same time accurate method of following the changes that accompany fatal injury. The material used in the most of these experiments was the marine alga, *Laminaria*.

The general result of Osterhout's studies was to show with many kinds of injurious agents a gradual fall of resistance, preceded in some cases by a preliminary rise, until a certain minimum was reached which was indicative of the complete death of the tissue. The whole course of the death change could be represented by a curve whose regularity and freedom from "breaks" showed that death does not occur at any given instant but is a progressive change, which may even be reversed in its earlier stages by a return of the tissue to normal conditions. Inspection of the curve obtained in a given experiment makes it possible to say at any instant how seriously the tissue has been injured and what will be its fate if it be restored to its original surroundings, i.e., whether it will recover completely, partially, or not at all. It must be remembered, however, in work of this sort that the point representing complete death is not absolutely fixed, since Brooks (1923) in a similar study on bacteria showed that it varies somewhat according to the method by which the organisms have been killed.

The increase in the permeability of cells caused by injury and death may also be studied by chemical methods. *Nitella* is especially well suited for investigations of this sort. Using it, Irwin (1923*a*) found that injury is promptly followed by exosmosis of chlorides which can be shown to begin at the point of injury and to spread from there along the length of the cell. Hoagland and Davis (1923) also state that exosmosis of chlorides is a very delicate method of determining injury in *Nitella*, while Osterhout (1923) still more recently has made quantitative comparisons on this material showing a very exact parallel between exosmosis and increase in electrical conductivity.

A special case of increased permeability resulting from injury, which is of much importance in physiology and medicine, is that of the capillary endothelium. Normally, the capillaries appear to be fairly freely permeable to water and to crystalloids, but to show a relative impermeability to colloids, which, to be sure, varies considerably in different parts of the body, and is different in the case of different colloids (Krogh, 1922). In the maintenance of the normal fluid balance of the body the osmotic pressure of the blood colloids (which can be effective only in the presence of a suitable semi-permeable membrane) plays an important part. If, therefore, while other factors such as blood pressure, etc., remain the same, the permeability of the capillary walls become increased, the colloids are no longer so effective osmotically as before, and liquid in excess of the normal amounts will escape from the blood, giving rise in extreme cases to a visible condition of oedema. Such an increase in the permeability of the capillaries may apparently be brought about by a variety of injurious agents such as heat, irritating chemical substances, mechanical injury, lack of oxygen, etc.

2. *Stimulation:*

It is frequently stated that stimulation of irritable tissues brings about in their cells a condition of greater permeability, and some of the more important theories of irritability postulate a mechanism by which sudden increases in permeability can be produced and propagated. As to actual experimental evidence in favor of this view, it must be admitted that while a great variety of observed facts, whose cumulative effect is very impressive, all point in the same direction, the individual observations are, for the most part, not as decisive as might be desired. For example, the case of the sensitive plant, *Mimosa pudica*, is frequently cited as an example of increased permeability resulting from stimulation, the sudden escape of liquid from the cells in the pulvinus being interpreted as the result of a change in the permeability of the latter. However, another possible explanation is that through chemical changes within the protoplasm the concentration of molecules in solution is suddenly decreased, with a resulting upset in the osmotic equilibrium of the cell, and an escape of water. Two of the most recent workers on the subject (Blackman and Paine, 1918) rather favor this latter explanation, though their results show an increased escape of electrolytes at the time of stimulation, which may perhaps be connected with permeability changes.

Another case which has frequently been quoted is that of the larvae of the worm, *Arenicola*, in which the escape of pigment from certain cells occurs under conditions which are also associated with muscular contraction (Lillie, 1909). The chief objection to this example is that the escape of pigment appears to be a pathological phenomenon associated with overstimulation, and it is questionable how far conclusions drawn from it may be applied to normal cells. Crozier (1922*a*), who has investigated a different type of cell in which the penetration of acid can be studied at the same time as the outward diffusion of a pigment, comes to the conclusion that while stimulation increases permeability of the cells to nitric acid, there is no connection between entrance of acid and escape of pigment. It should, however, be remembered that the penetration of strong acids is itself a more or less pathological process, which has no necessary connection with normal physiological permeability.

Another method of determining the effect of stimulation on permeability has been used in the case of muscle by McClendon, who placed the thigh muscles of the frog between platinum electrodes in such a way that their electrical conductivity could be measured in either the resting or the stimulated state. In his various experiments, the conductivity was found to be from 6 to 28 per cent greater in the latter condition than in the former, indicating, therefore, an increased permeability to ions during stimulation. While McClendon used precautions to prevent his results from being affected by changes in the shape of the muscle, etc., and while his conclusions are probably correct, it must not be forgotten that the technical difficulties of an experiment of this sort are considerable and the possible sources of error numerous.

The increase in permeability of muscle under similar conditions has also been studied by Embden and Adler (1922), who have shown on stimulation of the gastrocnemius muscle of the frog an increased escape of phosphoric acid, which in general magnitude runs parallel with the intensity of the physiological changes in the muscle. They claim to have ruled out the possibility that the increased output of phosphoric acid is due to an increased formation of this substance within the muscle during stimulation, though their data on this point are not very full. The same workers and also Simon (1922) have shown by the physiological method an apparently greater permeability to potassium at the time of stimulation (see also Weiss, 1922).

As other cases in which increases in permeability may perhaps be associated with stimulation may be mentioned the observations of Mitchell, Wilson, and Stanton (1921) on the absorption of rubidium and caesium by stimulated and unstimulated muscle, as well as those of Garmus (1912) on the intake of dyes by gland cells under the influence of pilocarpine. However, in both of these cases there are other possible factors of importance, such as, for example, changes in the capillary circulation of the sort described by Krogh (1919*b*). Finally, reference may be made to the decreased electrical resistance of the skin of man or of other vertebrates during the stimulation of a cutaneous nerve or under the influence of various emotional states—the so-called psychogalvanic reflex (see Gildmeister, 1913, and others).

3. *Fertilization:*

The process of fertilization seems to be associated with an increased permeability to many substances, but while the various experiments bearing on this point are in fairly good agreement with one another, they are not individually as convincing as might be desired. For example, Lyon and Shackell (1910) studied the staining of *Toxopneustes* eggs with certain dyes before and after fertilization, and found that while with Bismark brown and neutral red no differences could be observed, there was with methylene blue and with dahlia a distinct difference in favor of the fertilized eggs, which they interpreted as indicating an increased permeability in the latter. To rule out the possibility that the fainter color of the unfertilized eggs was due in the case of methylene blue to partial reduction in the absence of the intense oxidative processes following fertilization, they tried the experiment of first staining unfertilized eggs and then fertilizing them. They state that such eggs kept the same faint color until they had developed into swimming larvae. However, the fact that the eggs, placed in sea water, kept their color for such a length of time is an indication that the stain must have been held in a very firm combination by some constituent of the cell, and gives rise to the suspicion that the difference between the fertilized and unfertilized eggs may have been one rather of ability to combine with the dye than of the latter to penetrate.

Several workers have given evidence of increased permeability to ions on fertilization, as shown by the electrical conductivity method. For example, McClendon (1910*a* and *b*), using *Toxopneustes* eggs, in one experiment found a decrease in resistance on fertilization from 595 to 455 ohms. A similar result was obtained by Gray (1913–16) with the eggs of several species of sea urchins. It must be recognized, however, that there are a number of serious difficulties in making conductivity determinations on material of this sort, and also that there is a possibility, as suggested by Heilbrunn (1920), that conductivity may be indirectly affected by changes in protoplasmic consistency through changes in the character of the spaces between the tightly packed eggs.

Another set of experiments which may be mentioned in this connection are those of Harvey (1911) on the penetration of alkalies. Harvey showed that if *Toxopneustes* eggs were stained with neutral red, the time required for N/320 NaOH to penetrate the unfertilized eggs was approximately twenty minutes. In the fertilized eggs this time fell to thirteen minutes, then gradually rose to the original figure, only to fall again at the time of the first cell division to seventeen minutes. While these results seem to parallel those already described, it must be remembered that NaOH is a substance which in the concentration used enters cells largely through its destructive action, and, consequently, that it is possible that the case in question is not one of simple physiological permeability.

4. *Anaesthetics:*

If stimulation is associated with increased permeability, then it might naturally be expected that narcosis or anaesthesia would be associated with decreased permeability, or at least with a condition of the cell which makes sudden increases impossible. That such is the case has frequently been assumed in connection with theories of narcosis. (See in this connection Winterstein, 1919, pp. 258–76.) On the whole, the experimental evidence seems to be in favor of such a conception. For example, Lillie (1912) found that the effects of sodium salts in increasing muscular contraction, and at the same time loss of pigment from *Arenicola* larvae could be prevented by various anaesthetics; Tröndle (1920) that the entrance of salts into plant cells could be slowed or even completely prevented in the same way; Collander (1921) that anaesthetics check the penetration of acid dyes into plant cells; Crozier (1922*a*) the same in the case of the penetration of hydrochloric acid into the pigmented cells of *Chromodoris;* and Winterstein (1916*b*) also the same for the diffusion of salts through frog's muscle, etc.

The most satisfactory evidence of this effect, however, is found in the work of Osterhout, who by the conductivity method has been able to treat the subject quantitatively. For full details Osterhout's book (1922*c*) may be consulted, but it may be mentioned here that the first effect of a moderate concentration of a number of the commoner anaesthetics was found nearly always to be an increased electrical resistance of the tissues. This initial rise was then followed by a fall to the starting-point and finally below the latter until, if the experiment were sufficiently long continued, the limiting value characteristic of death was reached. It is very significant that in Osterhout's work the effects were reversible in all cases up to the time when the resistance had fallen to its initial value; after a further fall, except in the case of the relatively non-toxic ethyl alcohol, recovery was no longer possible.

If the concentration of the anaesthetic employed be sufficiently high, there is in *Laminaria* a fall in resistance from the start. With certain other types of material and other methods, no indications of decreased permeability have been obtained even over a considerable range of concentration, the effects being only those associated with an increase. Thus, Medes and McClendon (1920) obtained an increased exosmosis of chlorides from the leaf cells of *Elodea* with all the concentrations of alcohol, ether, and chloroform used. However, their method was not one which would show the actual course of the changes from minute to minute as does the conductivity method, but only the net result at the end of a much longer time, so that there is no necessary conflict between their results and those already mentioned.

5. *Hydrogen and hydroxyl ions:*

The manner in which H and OH ions may affect cell permeability depends on a number of possible factors which have not been very clearly distinguished in the past. The first is one which primarily has nothing to do with the cell at all, but rather with the penetrating substance. As an example, such an alkaloid as quinine may be chosen. It was shown by Overton (1896) that alkaloids in general penetrate cells readily while their salts (e.g., the hydrochloride or sulphate) do not. If, therefore, alkali gradually be added to a solution of quinine hydrochloride, more and more of the readily penetrating free base will be liberated; conversely, if acid be added to a solution containing some of the free base, the latter will combine with the acid and penetration will, in consequence, be less rapid. That the penetrating power and the physiological effect of alkaloids does largely depend on the reaction of the medium is shown by such work as that of Overton (1896) or of Tröndle (1920), though this factor did not appear to be of great importance in some experiments made by Boresch (1919) on a different sort of material.

The same condition is found with all substances which in the salt form penetrate with difficulty and as free acid or base with ease. This is true of ammonium salts which are all hydrolyzed to an extent which depends on the weakness of the acid combined with the ammonia and on the reaction of the medium. The entrance of free ammonia into cells from solutions of ammonium salts has been studied by Overton (1898) and by the author (Jacobs, 1922*a*), both of whom found a dependence of the rate of entrance on the reaction of the external solution. A similar case, but one in which penetration is favored by an acid rather than an alkaline reaction is that of the carbon dioxide-bicarbonate system (Jacobs, 1920*a* and *b*).

It is possible that with dyes the same principle may hold. Thus, Robertson (1908) showed by partition experiments between water and oil, and water and esters, that an acid reaction favors the passage of an acid dye (i.e., in the form of its free acid) into the non-aqueous phase, while an alkaline reaction has the reverse effect, the dye in such a solution existing in the form of a salt. With basic dyes, on the other hand, an acid reaction hinders and an alkaline reaction favors passage into the organic liquid. Harvey (1911) made experiments on *Spirogyra*, etc., whose results he explained in this manner. In her most recent papers, Miss Irwin (1922, 1923*b*), however, has given reasons for believing that in the case of the intake of brilliant cresyl blue by the cells of *Nitella* another explanation must be accepted, which depends on the principle which will next be mentioned.

An entirely different way in which H and OH ions might affect cell permeability is through their effect on the cell proteins. It is now well established (see Loeb, 1922*b*) that on the acid side of its isoelectric point a protein combines only with anions; on the alkaline side only with cations. It is apparent, therefore, that if the proteins are at all concerned in the intake of ionized substances into cells, the reactions of the medium and of the cell ought to exert a profound influence on this intake. Whether or not a given ion could be exchanged for one already combined with a cell protein and so gradually find its way into the interior of the cell might, therefore, conceivably depend on the relative concentrations of hydrogen and hydroxyl ions. This is the principle involved in the views of Bethe (1922) and of Irwin (1923*b*) in regard to the penetration of cells by dyes.

A third possible factor is indicated by the phenomena of electro-endosmosis mentioned on page 131. It has been shown by Loeb that with protein membranes the charge carried by the walls of the pores depends on the reaction of the medium (through the effect of the latter on the Donnan equilibrium). Since the movements of water and of substances dissolved in it are governed by this charge, the possibility of an indirect effect of H and OH ions on cell permeability is thus clearly indicated. A beginning in the study of electrical effects of this sort in connection with the penetration of the cell by electrolytes has been made by Girard (1910), some of whose results will be found in the paper cited. The general importance of the electrical charges of cell membranes and the effect on the latter of certain non-electrolytes is also discussed by Haynes (1921). The subject, as a whole, is much too large, and as yet much too unsettled, to be discussed in a satisfactory manner here, but it is, in the opinion of the writer, one of the most promising fields for investigation in the entire field of cell physiology, and is likely in the future to throw much light on many of the as yet unsolved problems of cell permeability.

6. *Other ions:*

In addition to H and OH ions it is known that many other ions profoundly affect the penetration of the cell by diffusing substances. Studies on the effects of electrolytes on this process are so numerous that reference is possible only to a few typical ones. Very instructive, because of their quantitative nature, are the experiments of Osterhout on *Laminaria*. (For a full bibliography see Osterhout, 1922*c*.) Among other facts brought to light by these experiments was the important one that certain cations such as $Na^{\cdot}$, $K^{\cdot}$, $NH_4^{\cdot}$, $Cs^{\cdot}$, and $Rb^{\cdot}$ tend to cause an increased conductivity, i.e., permeability to ions, while others such as $H^{\cdot}$, $Ca^{\cdot\cdot}$, $Ba^{\cdot\cdot}$, $Sr^{\cdot\cdot}$, $La^{\cdot\cdot\cdot}$, $Al^{\cdot\cdot\cdot}$, etc., have on the whole—though with limitations—the opposite effect. Thus, in one experiment (1912) disks of *Laminaria* placed in a pure solution of NaCl of the same conductivity as sea water showed at the intervals indicated the following resistance in ohms:

Beginning of the experiment	1,100
After 5 minutes	1,000
After 10 minutes	890
After 15 minutes	780
After 60 minutes	420
End of the experiment	320

In a solution of $CaCl_2$ of the same conductivity, the effect was very different. There was a preliminary rise during the first fifteen minutes from 1,100 to 1,750 ohms; this resistance was maintained for several hours and then there was a gradual fall, ending at the same point as in the preceding experiment.

It would be expected that if one group of cations (alkali metals) cause an increase in conductivity and another group (alkali earths) cause a decrease, then by combining representatives of the two groups in the proper proportions, the cell could be preserved in its normal condition. Such is the case. *Laminaria* tissues placed in a mixture consisting of 1,000 c.c. of M/1 NaCl plus 15 c.c. of M/1 $CaCl_2$ diluted to the resistance of sea water showed no change in twenty-four hours. It is significant that the ratio Na : Ca found to be most favorable in such experiments does not differ greatly from that in which these elements occur in sea water.

The available information about the permeability-increasing effect of salts of the alkali metals and the antagonistic effect of salts of the alkaline earths is by no means limited to facts obtained by the conductivity method, since numerous data of similar nature have existed in the literature for many years. Thus Loeb (1911) had suggested the probability of an increase in permeability as the result of the action of pure NaCl solutions, while Harvey (1911) had found the same effect in the case of the penetration of NaOH into cells of *Spirogyra* stained with neutral red. For example, in one of Harvey's experiments N/40 NaOH required ten minutes to enter the cells; in the presence of N/10 NaCl, however, the time required was only fifteen seconds, while with a certain mixture of NaCl and $CaCl_2$ it was three minutes.

Cases of decreased permeability from the effects of Ca salts are both numerous and striking. Loeb (1912) showed that if *Fundulus* eggs are placed in concentrated sea water they will float and remain alive for many days. If, instead of sea water, a solution of pure 1.5M NaCl be used, they quickly shrink in the hypertonic solution and sink to the bottom of the vessel. If, however, to the same solution of NaCl, $CaCl_2$ be added in the proper proportions, the eggs continue to float for days in an uninjured condition, neither water nor salts passing through the egg membrane in appreciable amounts. It is to be noted that $CaCl_2$ in great excess, e.g., in pure hypertonic solutions also increases permeability and the eggs sink, though not so rapidly as in pure NaCl.

Examples of the effect of calcium salts on the permeability of plant cells are also well known. True (1914) and True and Bartlett (1915*a* and *b*, 1916) have studied very carefully by the conductivity method the intake of electrolytes by the roots of lupine seedlings, as well as the reverse process of the leaching-out of salts from the roots into the external solution. They found that, in general, this leaching is very great in distilled water and fairly great in solutions of NaCl, KCl, etc., but that it is promptly checked by calcium, and, to a lesser extent, by magnesium salts. True (1922) also cites experiments by Eckerson in which the loss of sugars, etc., from roots and the penetration into the same tissues of copper sulphate were much more rapid in the absence of calcium than in its presence. It is to be noted, however, that the effect of calcium on plant roots is not merely one of making penetration difficult in all respects; as a matter of fact, in the absence of this element roots are unable to take up salts from the surrounding medium, i.e., they appear in this respect to be less permeable, though it is evident that the factors concerned in such a case are of a highly complex nature (True, 1922).

In the mammalian body a number of cases have been reported of a permeability-decreasing effect of calcium. Thus, according to Laqueur and Magnus (1921) calcium salts diminish exudation into the lungs after phosgene poisoning, and according to Rosenow (1916) they retard the passage of dyes from the blood into the aqueous humor, etc. R. Hamburger (1922) has also reported that artificial oedema may be produced by solutions deficient in calcium.

It would be convenient for the physiologist if he could remember as a general principle that Na, etc., increase, and Ca, etc., decrease cell permeability under all circumstances; however, conditions are not so simple as this. For example, Loeb (1922*a*) has recently investigated the effect of various salts on the penetration of *Fundulus* eggs by acids, and has found that the effect of Na is qualitatively similar to, though quantitatively less than, that of Ca and La, all of these ions to varying extents hindering the entrance of the acids. The same ions, however, favor the entrance of alkalies, so it is evident that "permeability" must not be looked upon as a simple property of the cell membrane which applies to all substances in the same manner. In addition to these effects on acids and alkalies, Loeb also reports in the same paper that Na also favors the penetration of cells by potassium, and points out the possible connection between this fact and differences in the distribution of the two elements such as those referred to on page 119.

7. *Miscellaneous substances:*

There are a number of other compounds belonging neither to the class of the electrolytes nor to that of the anaesthetics which have been shown to have an effect on cell permeability. Thus, according to Osterhout (1919*a*), caffein and the alkaloid cevadine have a permeability decreasing action much like that of calcium, and like it they may be used to antagonize the action of sodium. Morphine may also be used in the latter manner, and bile salts, which are electrolytes of a somewhat peculiar nature, behave similarly (Osterhout, 1919*b*). Szücs (1913) as the result of the following experiment concludes that hydrogen peroxide increases cell permeability to iron salts. If *Spirogyra* cells be exposed first to ferrous sulphate and then to H_2O_2, nothing happens; if, however, the order of exposure be reversed, a blue iron-tannin compound is formed. Szücs considers that the effect of light on plant cells described by Lepeschkin (1909) and by Tröndle (1910) may perhaps be brought about indirectly through the formation of hydrogen peroxide. However, it is possible to interpret his observation in other ways than that mentioned. In the case of a number of other substances, whose effects on cell permeability have been reported, the facts are even more doubtful. For example, Fluri (1909) thought that he had demonstrated a permeability-increasing effect of aluminium salts, but Szücs (1913) later showed that the failure to obtain plasmolysis which he observed was due to a solidification of the protoplasm and had nothing to do with permeability. Perhaps the same factor is concerned in the work of Krehan (1914) on KCN, since Heilbrunn (1920) has shown that this substance may likewise cause a reversible gelation of protoplasm.

8. *Temperature:*

The influence of temperature on cell permeability is of importance, not merely for its own sake, as a factor which profoundly influences all sorts of vital processes, but in connection with general theories as well, since from the magnitude of this effect on a given process it is frequently possible to draw conclusions as to the nature of the latter. In the case of cell permeability, temperature coefficients have been obtained by a number of workers, and while their magnitude varies greatly in different cases, they are in general high. Thus, Krabbe (1896) found that the rate of intake of water by pith cylinders of *Helianthus* was approximately five times as rapid at 25°C. as at 1°–2°C.—though it is evident that in cases of this sort factors other than permeability may be involved. Delf (1916) found for the same process in the scape of the dandelion and in onion leaves a temperature coefficient of from two to three for a rise in temperature of 10°. Stiles and Jorgensen (1915*b*) obtained for the penetration of HCl into potato tissue a temperature coefficient of approximately two, and Crozier (1922*b*) one ranging from 1.19 to 1.89 for the rate of entrance of HCl into the pigmented cells of *Chromodoris*. Masing (1914) found a high temperature coefficient for the penetration of blood corpuscles by sugar.

It has already been mentioned (p. 122) that the temperature coefficients for the entrance of at least some of the more readily penetrating organic compounds are low, i.e., of the order of magnitude characteristic of diffusion processes rather than of chemical reactions.

In contrast to the relatively high temperature coefficients for electrolytes mentioned above is one of only 1.33 obtained by Osterhout (1914) for conductivity in *Laminaria*. This value is, to be sure, higher than that of 1.26 found for sea water alone, but the difference is not very great. However, it would scarcely be expected that the normal intake of ions into cells and their movements under the influence of the electric current would be processes of the same nature, and it is not surprising, therefore, that their temperature coefficients appear to be different.

9. *Light:*

One other factor of considerable importance to botanists is light. This factor was investigated with much thoroughness by Lepeschkin (1909) and later by Tröndle (1909, 1910), who found that, in general, illumination appears to make the cell more permeable. Both Lepeschkin and Tröndle considered that their results had considerable theoretical significance in connection, not only with the question of the movements of plants, but with that of the translocation of food materials as well. Thus, in an illuminated cell, sugar would escape and so make further synthesis possible. The effects of light are, however, not of a simple nature, for Tröndle (1910) showed that they depend on the intensity of the light, the duration of the exposure, etc., and that there are certain characteristic after-effects, etc. Blackman and Paine (1918) also, using the exosmosis of electrolytes as their criterion, concluded that while light in general increases permeability, the same result may at times be brought about by a change from light to darkness. It is uncertain, therefore, how far effects attributed to light are actually specific and how far they may involve the larger problem of stimulation in general.

IV. THEORIES OF CELL PERMEABILITY

With so many of the facts regarding the penetration of the cell by diffusing substances still in uncertainty, the time is not yet ripe for attempting a comprehensive theoretical explanation of the process itself. Nevertheless, hypotheses are so necessarily and so inextricably connected with the acquisition of new facts that a review such as the present one would not be complete without some mention of several of the chief theories of cell permeability which have been suggested in the past, together with a brief criticism of some of the deficiencies of each. In general, it may be said that no single theory is entirely satisfactory, as indeed would be expected from the complicated nature of the facts which they attempt to explain. At the same time, there are probably elements of truth in most of them, and if each were regarded by its supporters merely as an attempt to deal with a limited number of the factors concerned in a very complex process rather than as a complete explanation of the behavior of the cell, there would be far less occasion for criticism than actually exists.

The majority, though not all, of the theories of cell permeability presuppose a plasma membrane of some sort, which exhibits a differential permeability, permitting some substances to enter the cell with ease (alcohol, ether); others with difficulty (most salts, sugars, etc.); and still others not at all (most colloids). Such a membrane was postulated by Pfeffer (1887) to account for his osmotic results, and has been accepted as a matter of course by most subsequent workers. However, there have not been lacking those who have attempted to explain cell behavior in other ways. For example, Moore and Roaf (1907, 1908) and Moore, Roaf, and Webster (1912), as the result of observations on blood corpuscles and on the behavior of various artificial membranes, came to the conclusion that the distribution of salts inside and outside of cells could be explained better by an adsorption hypothesis than by the usual membrane theory. M. Fischer (1915) also, in connection with his studies on oedema, has supported the view that the volume changes which occur when living cells are placed in various solutions are to be considered as cases of colloidal swelling, having nothing to do with semi-permeable membranes, the importance of which in such processes he denies.

While it must be admitted that a limited number of the peculiarities of cell behavior can be explained fairly plausibly by the foregoing hypotheses, it seems evident to most workers that mere chemical combination, "adsorption" and "colloidal swelling," are not sufficient to account for many well-known facts. For example, in the case of the red blood corpuscles, it is perhaps conceivable in the absence of other facts that the large amount of potassium in the cell might be retained by a combination, chemical or otherwise, with some cell constituent (though the work of Höber already mentioned on page 118 indicates that this is not so). But it is impossible to explain in this way the failure of sodium to enter the corpuscles from the plasma, since it is definitely known that this element exists in the blood chiefly in the form of freely diffusible ions. Furthermore, the high potassium content of the sap vacuole of *Valonia* and *Nitella* could not possibly be accounted for in this manner, since the studies of Osterhout (1922*a*) and of Hoagland and Davis (1923) have shown that the salts in the cell sap exist chiefly in an uncombined form. Finally, the practical absence of sulphates in the cell sap of *Valonia* (Wodehouse, 1917) according to this theory would have to be accounted for by "adsorption" by something in the surrounding sea water which thus prevents its inward diffusion. Such an explanation would not only be in conflict with the known facts of the chemistry of sea water, but would leave entirely unaccounted for the inward diffusion of these substances as soon as the cell is injured (Wodehouse, 1917). It is obvious, therefore, that the chemical combination or adsorption principles fail to provide an explanation for the differences between the salt content of the cell and that of its surroundings.

It might be thought that the Donnan equilibrium would provide a possible means of escaping from the membrane theory (see Roaf, 1912), but this is apparently not the case. As already pointed out, the Donnan theory demands that the ratio, Na (outside) : Na (inside) be equal to that of K (outside) : K (inside) as well as to that of Cl (inside) : Cl (outside), etc. This is very far from being true in the cells which have as yet been investigated. Certainly, in its present form, without the introduction of new principles, the Donnan equilibrium alone cannot account for the characteristic distribution of electrolytes inside and outside of cells, and still less is it able to account for the same differences in the distribution of non-electrolytes, such as sugars, etc. For the present, therefore, the membrane hypothesis seems to be the one with which the known facts are best in accord.

In the case of M. Fischer's observations, it is not at all difficult to see the inability of the theory of colloidal swelling to account for the volume changes of cells in various solutions. In the first place, it is usually true that cells of both plant and animal origin, when placed in a great variety of solutions of electrolytes or slowly penetrating non-electrolytes of the same freezing-point, are similarly affected as to their volume. If the same series of solutions be used with gelatin or fibrin, however, the effect produced will be found to bear no relation to the freezing-points of the solutions in question. It may be the same in two solutions of very different freezing-points, or utterly different in two of the same freezing-point. The factors concerned here are evidently very different from those in the preceding case, being, in fact, those on which the Donnan equilibrium depends. In the second place, if cells and granules of gelatin, stained with neutral red or some other suitable indicator, be subjected to the action of very weak solutions of, for example, HCl or NaOH (pH from 5.0 to 9.0), the acid or alkali will be found to enter the gelatin granules immediately, producing at the same time characteristic volume changes; in the case of the cells, however, though their much smaller size and relatively greater surface might be expected to favor penetration, internal effects either on the indicator or on the volume of the cell may be completely absent or at least be delayed for a long time, indicating some hindrance to diffusion. Many other examples could be given which would show the imperfect nature of Fischer's analogy, but those cited are perhaps sufficient. On the whole, therefore, it seems impossible to escape from some form or other of Pfeffer's view as to the rôle of semi-permeable membranes in the osmotic phenomena in cells.

Other independent lines of evidence favor a belief in the presence of such membranes. For example, cells have a fairly high electrical resistance which is certainly not due to the absence of ions capable of conducting the current. This resistance is greater across a tissue such as muscle than in the direction of the fibers; it decreases with injury and death in a manner which parallels the power of substances in general—even of non-electrolytes—to enter and leave the cell; and it is less with alternating currents of high than of low frequency, the significant difference in the two cases being in the length of the paths traversed by the ions. All of these facts find their most plausible explanation on the membrane hypothesis. For a further discussion of other electrical properties of cells in their relation to membranes, see Section IV.

There may also be mentioned the evidence in favor of a plasma membrane obtained by the micro-dissection and micro-injection methods, which are more fully discussed in Section V. Attention may be called at this point merely to a few facts such as the following: According to Chambers (1922) an aqueous eosin solution does not stain *Amoeba* from the exterior; if injected into the interior of the cell, however, it spreads through the protoplasm. Likewise, Lillie, Clowes, and Chambers (1919) found that an old solution of mustard gas (which contains free HCl) is relatively harmless to the egg of the starfish until injected into the interior of the cell when its destructive effect is immediately apparent. Finally, the differential effect of the free NH_4OH and HCl present in a solution of NH_4Cl is obtained only when diffusion is allowed to take place through the cell membrane. When the solution is injected into the interior of the cell this effect is entirely lacking, as is also the case with a mixture of CO_2 and $NaHCO_3$ (Chambers, 1922).

It may be considered, therefore, as fairly well established that a living cell is provided with a membrane (or at least with an external region which may perhaps be of more than filmlike thickness) whose properties are different from those of the remainder of the protoplasm, and which is to a great extent concerned in limiting and modifying the simple process of diffusion. As already mentioned, the various theories of cell permeability presuppose such a membrane; they differ sharply from one another, however, with regard to its probable nature.

The theory which has played a greater part in the past in discussions of cell permeability than any other is the lipoid theory of Overton. (For a statement of it in this author's own words see Overton, 1902, p. 264.) The grounds on which it is based are several, of which the following are the most important: first, the non-miscibility of protoplasm with water, which can plausibly be accounted for by the presence of a film of some sort of fatty material; second, the fact that the lipoids are substances which have a high degree of "surface activity" and which might, therefore, reasonably be expected from the Principle of Gibbs to collect at free surfaces, automatically repairing injuries, etc.; and third, the almost perfect correlation which Overton found between the lipoid solubility of hundreds of organic compounds and the ease with which they enter cells.

As to the nature of the fatty material, Overton considered that it could not be an ordinary fat, partly from the impossibility of producing saponification of the cell membrane with, for example, 2 per cent Na_2CO_3, and partly because of the impermeability which such a film might be expected to show to water. He was inclined to believe that it consisted of a mixture of lecithin and cholesterol, both of which are known to be of very wide distribution in cells, and the first of which has considerable powers of absorbing water. Overton did not state, as many have thought, that the film consists entirely of lipoids, but only that it is impregnated with them. He also considered that these substances might be found in the interior of the cell as well as at its surface.

In the light of our present knowledge, Overton's hypothesis certainly went too far in its overestimate of the difficulty of entrance into the cell of lipoid-insoluble materials such as sugars, salts, etc.; in fact, as already mentioned, some of the observations on which he based this part of the theory were almost certainly in error; and such substances are now universally admitted to enter cells. Furthermore, he was unsuccessful in attempting to reconcile the intake of water with that of lipoid-soluble substances by the supposition that lecithin is the material chiefly concerned in determining cell permeability, since as Nathansohn (1904*b*) has pointed out, water-soaked lecithin no longer retains its original solvent power for typical lipoid-soluble substances but behaves more like water itself. Overton's theory in its original form is, therefore, evidently unsatisfactory in at least these two respects. But that there is some relation between the ease of entrance into the cell of organic compounds and their lipoid solubility is indicated, not only by Overton's own very extensive observations, but by a great many facts which have been brought to light by others. The supposed exceptions in the case of lipoid-soluble dyes are, as previously mentioned, less numerous than some have supposed, and very few facts are known which even appear to conflict with this part of the theory.

At this point it may be mentioned that another interpretation has been made of certain of the facts observed by Overton and others. If substances be classified, not according to their lipoid solubility, but according to their power of lowering the surface tension of water, it will be found that there is a very similar relation between this property and their ability to enter cells. In the case of the fatty acids, for example, not only does relative lipoid solubility increase with increasing length of the hydrocarbon chain, but surface activity does as well; indeed, these properties very frequently run parallel. Facts such as those mentioned have been used by Traube (1904) as the foundation for a new theory of cell permeability, the "Haftdruck" or "Retention Pressure" Theory. Stated in simple terms, this theory is that a substance which has a low "Haftdruck" in water (i.e., a low affinity for it) will tend to leave it and to accumulate at its free surfaces. Such a tendency will result in a large collection of the substance at cell boundaries with a greater tendency in consequence to diffuse into the cells.

This was the theory in its original form. When it later became apparent that the degree of accumulation at the interface: protoplasm-water, depends not merely on the affinity of the substance for water (as indicated by the smallness of its effect on the surface tension of a water-air boundary), but on that of the protoplasm as well, Traube extended the hypothesis to take account of the "Haftdruck" in the protoplasm also (1910). In its revised form, the theory states in effect that when the "Haftdruck" in water is low and that in the protoplasm high the substance will enter the cell, but not when conditions are reversed. However, in this form, the theory becomes very similar to that of Overton, since "Haftdruck," "affinity," and "solubility" are all more or less related ideas, and are what are really fundamental to both theories, surface tension effects being involved only indirectly as an indication of the "Haftdruck" itself.

When objections to the lipoid theory of Overton began to accumulate, various workers in search of an alternative explanation of the non-miscibility of protoplasm with water saw a possibly analogous case in the observation of Ramsden (1904) that white of egg and other proteins tend readily at free surfaces to form a "haptogen" membrane of solidified protein. It was supposed that certain of the protein constituents of protoplasm have a similar tendency, even being able automatically to repair breaks in the surface film and so to prevent mixing of the protoplasm and the surrounding liquid. The haptogen membrane theory never attained the prominence nor excited the same amount of discussion as the lipoid theory, and few workers have cared to commit themselves to it in as simple a form as that just mentioned; nevertheless, many observations have accumulated which have been interpreted by those who made them as indicating the importance of proteins in the structure of the plasma membrane and in questions of cell permeability. (See, for example, Robertson, 1908; Osterhout, 1911; Lepeschkin, 1910; Loeb, 1911; etc.) However, the haptogen membrane theory, in its simplest form appears to be entirely inadequate to account either for the great variability in the behavior of living cells with respect to diffusing substances, or for certain specific facts such as the extraordinarily ready penetration of non-polar, lipoid-soluble substances. If the lipoid theory, as originally advocated by Overton, breaks down because it fails to explain the entrance into the cell of the salts discussed on pages 118–25, so the haptogen membrane theory also breaks down because it is unable to account for the peculiar behavior of the various organic compounds mentioned on pages 114–18. Furthermore, it is possible to test the permeability of artificial haptogen membranes to selected substances and to compare this with the permeability of cells to the same substances. Harvey (1912) has made a comparison of this sort in the following manner. If a solution of lecithin in chloroform be shaken up with dilute white of egg, chloroform droplets are formed, each surrounded by a film of protein. On standing, the chloroform gradually passes out into the water, and water mixes with the lecithin, until an aqueous suspension of the latter within the membrane results. The droplets, which now bear a striking resemblance to certain ova, may be stained with neutral red and used for studying penetration by acids and alkalies. Using this method Harvey showed that while in the case of living eggs NH_4OH penetrates far more rapidly than NaOH, in the case of the "artificial cells" the rate of penetration is approximately equal in the two cases. In other words, a haptogen film of protein has not the same properties as a natural cell membrane. Both Loeb (1912) and Crozier (1916*c*) also, in discussing questions of cell penetration, while emphasizing the probable importance of proteins in the process, point out that lipoids seem to be concerned as well.

It appears, therefore, that neither a lipoid membrane nor a protein membrane alone is capable of explaining all of the various peculiarities of cell permeability. In an attempt to obtain the advantages of both theories without the limitations of either one alone, Nathansohn postulated a membrane composed of a mosaic of both lipoids and proteins. Through the lipoidal portion of such a membrane, substances like ether, alcohol, etc., could enter unhindered while through the remaining portions other substances could enter in a manner which might vary considerably from time to time according to hypothetical changes in the proteins. The chief objection to the Nathansohn theory is that it is purely a speculation, supported merely by the negative evidence that the same objections cannot be offered to it as to the other theories previously mentioned.

Within recent years, however, a somewhat more definite conception of the manner in which both lipoidal and aqueous phases might coexist at the cell surface has been put forward by Clowes (1916). Using the principle that the soaps of calcium, barium, strontium, etc., are more soluble in oils than in water while those of sodium, potassium, etc., show the reverse relation, Clowes showed that he could secure at will, from a given mixture of oil and dilute alkali in water, an emulsion of oil in water or of water in oil, by adding to the mixture different amounts of sodium and calcium ions. An excess of calcium gave the second type of emulsion; an excess of sodium the first. By combining the two elements in the proper ratio a point could be reached where the opposing tendencies balanced each other and the two phases separated into layers. The critical ratio of Na:Ca, on one side of which one type of emulsion exists and on the other side of which the other type exists, is not very different from the ratio in which these elements occur in sea water or in the body fluids of most animals.

It may readily be imagined that if protoplasm possess an emulsion-like structure—a conception held by many previous workers—the latter would likely be definitely of neither one type nor the other, but would tend to vary in different parts of the cell and even from moment to moment in the same part. If, to such a system, a calcium salt were added, the type of emulsion would be favored in which the lipoidal materials were the external or continuous phase, and permeability to water and water-soluble substances would be decreased. On the other hand, salts of Na, K, etc., by favoring the reverse type of structure would increase the permeability to such water-soluble substances. That effects of this nature are produced in living cells by the ions in question has already been mentioned (p. 145) and the theory of Clowes, therefore, has the great advantage that it accounts not merely for the entrance of the cell by both lipoid-soluble and lipoid-insoluble substances, but for certain characteristic effects of electrolytes on cell permeability as well. For further analogies between emulsions of this sort and living cells, with respect to the action of anaesthetics, etc., the original paper of Clowes must be consulted.

One further point remains for discussion: namely, the effect on penetrating power of the size of the molecule. That this may at times be a factor of importance is shown by the failure of the majority of proteins—even those which form true molecular solutions—to enter most cells. The same thing is seen with many dyes of high molecular weight. It must not be thought, however, that below a certain point there is any correlation between molecular weight and difficulty of penetration; indeed, such a relation as does exist is frequently the exact reverse of this. For example, the fatty acids apparently enter cells with increasing ease as the molecular weight increases—at least up to a certain point. The ease of entrance of the alkaloids as compared with the simple inorganic salts is also another case in point. Evidently molecular weight alone has little to do with cell permeability except as a limiting factor when it exceeds a certain magnitude. It has been suggested by Bayliss (1920, p. 113) that perhaps the effective molecular weight of the slowly penetrating salts may be increased by hydration and that these substances may, therefore, be only apparent exceptions to the principle in question. However, too little is definitely known at present about hydration in solutions to make possible any decisive test of this hypothesis.

Of other theories of cell permeability, of which there are a number, nothing need be said here; the whole subject is of too speculative a nature to make further discussion profitable. In conclusion, it may be emphasized that what is most needed in the field of cell permeability at the present day is facts. When sufficient accurate quantitative data covering a wide range of material and based upon a sufficient number of independent methods have become available, a satisfactory theory will follow as a matter of course. Until that time, speculations should be reduced to a minimum.

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SECTION IV

REACTIVITY OF THE CELL

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REACTIVITY OF THE CELL

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All living systems, including single cells as well as complete organisms of all kinds, react to changes occurring in their immediate environment, or to changes in their relations to the environment, by exhibiting characteristic alterations in their own special activity. This power of *reacting*, of varying their activity in response to environmental change, is the fundamental physiological property to which we have applied the term "reactivity." We have chosen this term in preference to the more familiar term "irritability," as being more directly descriptive of the essential biological relationship. An isolated nerve is "irritable" but does not "react" in the sense of acting upon and changing the conditions in the surroundings. The ability thus to react on the surroundings is the essential and biologically important property whose basis we are about to discuss.

This kind of responsiveness is a fundamental attribute of living matter. We observe that almost any kind of environmental change, if its degree and rate are sufficient, may act as a "stimulus" to the living cell or cell system and call forth a "reaction" or "response." Mechanical influences (impact, contact, pressure), gravity, presence of special chemical substances, changes of temperature, light, electrical conditions, all are included in the list of stimuli. Changes in the *relation* of the organism to the environment, although the environment itself remains unchanged, may also furnish the conditions for stimulation and response; thus an animal moving from place to place continually encounters new conditions to which it reacts. In general we may say that effective stimuli—conditions eliciting response—represent in all cases changes in the relations between the irritable living system (cell or organism) and the immediate environment. By immediate we mean those portions of the external world which are in immediate contact with, or impinge directly upon, the living system. The cases where the cell or organism responds to influences coming from a distance represent no exception to this rule; thus rays of light coming from a distance stimulate the retinal cells, but this occurs only when they reach and penetrate the latter.

The living system differs from most inanimate systems in its relation to environmental change in being not simply passive but itself a source of energy; under appropriate conditions it releases a portion of this energy and performs work on the surroundings, i.e., reacts. Each living cell continually or intermittently receives energy (e.g., the potential chemical energy of foodstuffs) from the surroundings; later it returns this energy to the surroundings in a kinetic form, usually as mechanical energy or heat, in some cases as light or electricity. What is characteristic is that the rate and character of this interchange of energy are controlled or regulated by the activity of the living system itself.

Since, in the nature of things, living system and environment are always in contact with each other, any change in the immediate surroundings has necessarily some direct physical effect upon the living system. This effect (e.g., mechanical impact, local chemical or electrical change, etc.) furnishes the conditions for some special and often complex change in the activity of the living system. For example, when an infusorian or a *Hydra* is touched with a needle the animal executes a definite series of movements or "avoiding reaction." Evidently the direct physical change produced by the contact or "stimulus" is an event of an entirely different kind from the succeeding organic "response"; the latter is usually much more complex, and has special features depending on the inherited organization of the living system; yet the connection between the two is definite and constant. Such an instance illustrates the general nature of the stimulus-response relationship; the stimulus causes directly some change, which may be relatively very slight, in the living system; this change in some way initiates or releases a process (or series of processes) whose nature and extent are determined by the special organization and properties of the living system. This kind of relationship is often spoken of as a "trigger relationship"; its distinguishing feature is that the energy expended in the stimulus has no fixed relation to the energy set free in the response. The control which the environmental conditions exercise upon the processes in the living system is largely although not entirely one of this kind. The physiological activity of the system and hence its relations to the environment are thus regulated. We may compare this mode of regulation to that exhibited in a complex artificial mechanical or electrical system; valves and switches, operated with slight expenditure of energy, control the flow of much larger quantities of energy through the system and hence the work which the latter performs. Similarly, in the living organism the release of stored energies is determined and controlled by relatively slight changes in the condition of the system, caused by the direct action of the environment upon it.

It will be evident from this brief general discussion that the vital activities cannot be considered as determined apart from the conditions in the surroundings. In this sense, organism and environment form parts of a single system, exhibiting a complex type of equilibrium characterized by a continual interchange of materials and energy. Normally this interchange is of such a kind that the organism maintains itself in its environment—lives, grows, and eventually reproduces itself. Evidently, in order to accomplish this result successfully, its own activities must be *adjusted* to environmental processes and conditions; for example, survival requires that the animal should react positively to food or sources of food and to other conditions necessary to life, and negatively or in an avoiding manner to indifferent or injurious conditions. Such considerations explain why the reactions of living matter are characteristically *selective*, and survival depends directly on this selective quality; the special modes of activity and response exhibited by any organism must on the whole conduce to the continued existence, or survival in nature, of the individual or the species. Hence reactivity, as we meet it in intact organisms in a state of nature, usually impresses us by its adaptive character.

In this section our chief concern is with those general or fundamental protoplasmic properties upon which the responsiveness of all cells or cell systems to stimulation depends. Each living cell, e.g., an egg cell or an epithelial cell, as well as a muscle cell or nerve cell, is a reactive system, one whose physiological activity changes in response to changes in its environment. The precise character of the response varies from cell to cell and depends in each case upon special inherited features of structure and organization. For example, the unfertilized egg cell responds to the contact and entrance of the spermatozoön by beginning its cycle of cell division and development; the same response may be induced by an artificial activating agent. A stimulated gland cell secretes, a muscle cell contracts, a protozoön passes through an often complex sequence of motor reactions; a stimulated plant organ accelerates or otherwise changes its state of growth. Such instances illustrate the variety of response exhibited by different types of cell. But notwithstanding this diversity of detail, all show one fundamental property in common, namely, modifiability of the characteristic physiological activities under comparatively slight changes of external condition. Our task is to inquire into the general nature of the conditions determining this modifiability.

This property cannot be understood without taking into consideration the general physicochemical constitution and properties of living matter, or protoplasm, as distinguished from non-living matter. Living matter is characterized chiefly by its complex chemical constitution and structure and by the special nature of its relations with the environment. In all cases the living system incorporates certain selected materials and energy from the environment, and transforms them within the living substance in the special manner characteristic of the species. It is by means of this process of physical and chemical transformation, and by the accumulation of certain of its synthesized products, that the living system, with its specific composition and activities, is built up from the non-living materials of the environment. In the normal maintenance of any living system, the same processes of selective incorporation and specific synthesis are concerned. Side by side with the synthetic processes proceed the disintegrative or energy-yielding processes, chiefly chemical reactions of an oxidative kind; these furnish the energy by which the organism acts upon its environment. In all cases the process by which energy is freed is the chemical decomposition, usually oxidation, of protoplasmic constituents; these may be taken in bodily from the environment as food, or synthesized within the living cell, as in plants. The energy freed within the cell by oxidation is partly returned to the environment as heat or mechanical work, and partly applied in the internal work of the cell or in effecting special syntheses, e.g., those underlying growth. What is essential to note is that all cellular activities, whatever their special nature, are based upon processes of chemical transformation. The cell is primarily a metabolizing system.

The characteristic irritability and reactivity of protoplasm show that external agencies, when they act upon the cell, in some way modify these chemical reactions. Apparently the stimulating agent effects some change in the internal condition of the protoplasm, and as a result the velocity of the chemical reactions is altered, typically increased; increased growth, increased mechanical activity or heat production, and increased consumption of O_2 and output of CO_2 are evidences of this increase in reaction velocities. It is noteworthy that both the energy-yielding (or "katabolic") and the synthetic ("anabolic") reactions may be thus influenced; thus many plants respond to stimulating conditions by increased growth, implying an increase in the rate of synthesis of structure-forming materials. Conversely, a stimulated muscle cell performs mechanical work whose energy is derived from the oxidative breakdown of carbohydrate. A further fact of great general interest is that in many cells the energy-yielding and the synthetic reactions form a closely interconnected cycle; thus in voluntary muscle, increased contractile activity, involving increased oxidation of sugar, is followed by increased growth (functional hypertrophy); and in many other cases there is evidence that the oxidation of carbohydrates is a necessary condition for the syntheses underlying normal growth.

These general facts lead us to formulate the fundamental problem of protoplasmic reactivity as follows: What are those special features in the composition or constitution of living matter which render its chemical processes so susceptible to influence by changes in the surroundings? Living protoplasm, as we have seen, is a complex system, consisting of a large variety of chemical compounds associated in a special type of structure. Both its chemical composition and its structural or morphological constitution (arrangement of parts) are to be regarded as factors in the determination of its special type of activity. Now the purely chemical composition of protoplasm, considered by itself, does not sufficiently explain its special property of reactivity. The principal features of this composition have been described already in Section II. The chief organic compounds, proteins, carbohydrates, lipins (fats, lipoids), and related compounds, are substances of high chemical potential; they represent a large reserve of energy, which is freed when they are oxidized to CO_2 and water. In themselves, however, they are stable compounds, not readily oxidized at low temperatures at the oxygen-tension and H-ion concentration of the cell. The presence of the other organic compounds, including enzymes, and of the inorganic compounds (water, oxygen, salts) also does not sufficiently account for the readiness with which the energy-yielding chemical reactions occur in protoplasm, and for the variability in their velocity in irritable types of cell. We must conclude that reactivity depends on other conditions than the mere presence of these various compounds within the space of a single cell. The chief of these conditions is the special structural constitution or organization of protoplasm; apparently this structure is responsible for the special peculiarities of its chemical behavior. What we have chiefly to account for is the high velocity with which the characteristic chemical reactions, e.g., the oxidation of sugar or the synthesis of proteins in growth, proceed in the living cell. The special peculiarity of highly irritable cells is the variability of this velocity under changing external conditions; this property cannot be understood until the general physical conditions controlling the protoplasmic reaction velocities are first determined.

All of the evidence indicates that these conditions are intimately connected with the special *structure* of the protoplasmic system. The significance of the term structure as applied to protoplasm should first be defined. It is evident that a mere random mixture of cell constituents, in the same proportions and concentrations as in protoplasm, would not give a system having the properties of the living cell. The constituents must have a definite arrangement, and must be present in a definite physical state; only under these conditions is it possible to conceive of any kind of ordered interaction such as that underlying the life of the cell. Broadly speaking, by the term structure as used in biology we mean the permanent spatial distribution and physical state of the essential constituents of the living system; the term organization has a similar significance, referring especially to those permanent features of structure and composition which underlie or determine the specifically vital properties.

Protoplasm may be described as a chemical-reaction system in which the reactions are controlled by structural conditions. Sugar, for example, when introduced into the living cell, is oxidized rapidly because it is brought into a system having a special type of structure. Accordingly when we alter protoplasmic structure we alter the rate and in many cases the character of the reactions occurring in the system. As we shall see, there is much evidence that in the stimulation of any irritable cell, e.g., a muscle cell, the primary change is an alteration of structure, affecting the permeability and electrical polarization of the surface layer of protoplasm. A change of a similar kind is then apparently transmitted to the inner protoplasm and alters the chemical and other processes throughout the cell. It is known that many forms of protoplasm are structurally unstable, the whole cell rapidly breaking down when exposed to slight changes of mechanical or other conditions; the blood platelets and various other cells, e.g., leukocytes, explosive corpuscles of crustacea, nematocysts, illustrate this behavior (see Sec. V). Apparently in irritable cells a somewhat similar structural change, only reversible and less far-reaching in its effects, results from stimulation; this change determines secondarily the sequence of chemical and physiological processes constituting the response. According to this conception irritability depends on the peculiar instability or lability of protoplasmic structure. The probable conditions of this instability will be indicated later. We shall first briefly review the evidence that the chemical reactions of the cell are largely determined by structural conditions.

I. INFLUENCE OF STRUCTURE ON CHEMICAL REACTIONS IN THE CELL

It is a familiar fact that the structure of protoplasm undergoes profound alteration at death. In translucent forms of protoplasm one of the first changes to be observed is a coarsening or increased opacity, indicating coagulative changes in the cell proteins. Changes of physical consistency, death rigor, loss of tensile strength, increase of viscosity, are also frequent. Associated with these structural alterations are chemical effects of various kinds; the protoplasm frequently turns acid (in muscle from lactic acid); and autolytic or other enzymatic reactions are initiated, e.g., the oxidase reaction causing the browning of crushed fruits or tubers. The appearance of these reactions, coincidently with the structural alterations associated with death, is a general indication of the interdependence between structure and chemical reactions in protoplasm; many recent experimental studies have furnished more definite evidence of the same kind.

In the case of the vertebrate muscle cell the relation of chemical processes to protoplasmic structure is well illustrated in the work of Fletcher and Hopkins (1907, 1917) on the formation and disappearance of lactic acid. Lactic acid is formed in each act of stimulation; it also appears in dying muscle or under the influence of cytolytic agents (heat, chloroform, etc.) or as a result of mechanical disintegration. It is derived from the carbohydrate of the muscle cell (apparently from glycogen by way of hexose-phosphate); its formation is intimately connected with the normal functional activity of the cell, the present indications being that the acid causes an increase in the surface tension of the colloidal elements of the contractile fibrils, the contraction being the direct expression of this change. The power of spontaneously forming acid is present only while the muscle is irritable, i.e., alive; the same is true of the power of removing the acid formed in stimulation. The quantity of acid formed in frogs' muscle fatigued by repeated stimulation is 0.2 to 0.25 per cent of the total weight of muscle. In the intact living muscle, in the presence of oxygen, it disappears in a few hours, being apparently resynthesized in great part to carbohydrate; in an oxygen-free atmosphere it remains unchanged or increases in quantity. The oxidative process by which the lactic acid is removed is dependent on the normal protoplasmic structure; there is no disappearance of lactic acid in a muscle whose structure has been destroyed by cytolytic agents, heat rigor, or mechanical injury. As Fletcher and Hopkins (1907) express it, the maintenance of the normal architecture of the muscle cell is an essential condition for this effect of oxygen. A relation of the normal protoplasmic structure to the synthetic activity of the cell, as well as of this synthetic activity to oxidation processes, is thus indicated.

The fundamental relation of protoplasmic structure to oxidation processes is also shown in the experiments of Harden and Maclean (1911) and especially of Warburg (1914). Harden and Maclean found the oxygen consumption of isolated tissues to be greatly diminished when cell structure was mechanically destroyed by grinding with sand, and the more completely the greater the destruction; Warburg has made similar observations with many types of cell (tissue cells, yeast cells, bacteria, egg cells, blood corpuscles). He compared the oxygen consumption of sea-urchin eggs in the intact state, both unfertilized and fertilized, with that of the residue obtained by complete mechanical destruction in a mill with steel balls. The structureless cell residue thus obtained still consumes oxygen and evolves carbon dioxide, but at a much slower rate than the intact, especially fertilized, eggs; e.g., oxygen consumption is reduced to 10 per cent (or less) of that of the intact fertilized eggs. Disintegration of protoplasmic structure by freezing and thawing also greatly lowers the oxidative activity of the cell. Warburg has performed similar experiments with other cells, e.g., the erythrocytes of birds; when these cells are frozen and thawed the protoplasmic structure is broken down and the hemoglobin and other soluble cell constituents are set free. Such a suspension of destroyed cells still retains much of its original power of consuming oxygen, but this property is present only in those parts of the suspension which still contain remnants of the solid or structural portions of the original cells. By centrifuging the suspension of destroyed cells, two layers are separated, one containing the dissolved cell constituents, the other the residue of the solid cell structures. Oxygen consumption is confined almost entirely to the latter layer. Similar experiments on liver cells and sea-urchin eggs show that here also the solid elements of the cell (granules, etc.) are necessary for active oxygen consumption (Warburg, 1921).

Such experiments throw light on the conditions under which oxidations and presumably other chemical reactions occur in living cells. Apparently these reactions proceed most actively—although probably not exclusively—in contact with the solid or structural cell material, i.e., at the boundaries of the protoplasmic phases; in other words, the surfaces of membranes, fibrils, granules, and other solid cell structures have an accelerating or catalytic influence on these reactions. This is indicated also by certain microchemical reactions by which colored oxidation products are formed within the cell; for example, the formation of the blue dye, indophenol, by oxidation of a mixture of α-naphthol and p-diamino-benzene, occurs in the blood corpuscles of the frog most rapidly at the surface of the nuclear and plasma membranes (R. S. Lillie, 1913). These surfaces apparently catalyze the reaction, and similar effects are probably characteristic of other structural surfaces in cells. Other facts, especially the facts of electrical stimulation and of narcosis by surface-active substances, also indicate that the boundary surfaces of protoplasm have in general a special determinative or catalytic relation to the chemical reactions of the cell.

The acceleration of chemical reactions by contact with finely divided solid materials (charcoal, platinum, etc.) is well known in inorganic chemistry and constitutes the phenomenon of heterogeneous catalysis. There is every evidence that the structural surfaces in living cells exercise a catalytic influence of the same kind, although other factors (especially electrical factors and factors dependent on specific chemical relationships) also enter and render the conditions more variable and complex than in inorganic systems. There can be no doubt, however, that the high velocity of the more characteristic chemical reactions of protoplasm (e.g., the oxidation of sugars and amino acids) is chiefly determined, directly and indirectly, by the polyphasic structure of the living system. This condition implies the presence of a large surface of contact between the solid or structural elements of the cell system and the aqueous solution, containing the oxidizable compounds, which forms its continuous phase. Warburg's recent investigations on the oxidation of organic compounds under the influence of finely divided animal charcoal show many striking parallels with the protoplasmic oxidations, although the parallelism is not complete, e.g., the rapid oxidation of sugar has not yet been accomplished by this means. Amino acids, however (cystin, leucin, tyrosin), are rapidly oxidized; the same is true of other organic compounds, e.g., oxalic acid and alcohol (Warburg, 1914, 1921). When charcoal is added to an aqueous solution of cystin (the sulphur-containing amino acid of proteins) and shaken with air at room temperature, the oxygen disappears simultaneously with the cystin, while CO_2, H_2SO_4, and NH_3 are formed; i.e., the cystin is oxidized to the same end products as in the living cell. The rate of oxidation is also closely similar; e.g., a suspension of charcoal containing a solution of cystin (M/500) was found to consume as much oxygen in a given time as an equal weight of mammalian liver (Warburg, 1921, 1922). Charcoal may thus accelerate certain oxidations to the same degree as actively metabolizing living cells. Since amino acids in aqueous solution at neutral reactions are typically stable compounds, the fact that in a heterogeneous system of such simple composition they undergo rapid oxidation is a very clear indication of the important part which surface conditions play in the metabolic reactions of protoplasm. The suspension of charcoal may be regarded as a simple model illustrating the part played by surface conditions in cell metabolism. The resemblance becomes still more striking when the action of surface-active or narcotizing compounds on these oxidations is studied; small quantities of alcohols, nitriles, amides, and similar compounds retard the oxidations in charcoal suspensions to about the same degree as they retard oxygen consumption in living cells (e.g., blood corpuscles). Another remarkable parallel is that slight traces of iron salts greatly accelerate the catalytic oxidation of amino acids by charcoal, just as they accelerate oxygen consumption in certain animal cells, e.g., sea-urchin eggs. Cyanide, on the other hand, depresses oxidations both in the charcoal system and in living cells (Warburg, 1921, 1922).

These and similar results are of much interest as showing that a simple type of structural condition, namely, the presence of finely divided water-insoluble material at whose surfaces adsorption may take place, may promote, or catalyze, many chemical reactions of the kind characteristic of living matter. The structure of living matter is, however, much more complex than that of a suspension of charcoal or platinum; and correspondingly many reactions occur in the cell which are not catalyzed by inorganic catalyzers, so far as investigation has yet shown. This is true of the oxidation of sugar, and especially of the synthetic reactions determining growth, repair, and maintenance. These reactions appear to be highly sensitive to adverse conditions, especially to alterations of structure; it is remarkable that, while many of the other reactions of living matter, such as oxidations and hydrolyses, proceed rapidly in dead or structurally disorganized protoplasm (as shown in autolysis), the synthesis of new proteins, the most characteristic chemical reaction of living protoplasm, ceases or becomes inappreciable. It is probable, however, that in these reactions other factors enter than those ordinarily classed as catalytic, e.g., electrical factors and others of a kind associated with stimulation. These factors appear to be operative only while the "living" protoplasmic structure remains intact. All of the evidence indicates that the power of synthesizing new compounds depends on the same conditions as those determining irritability.

II. GENERAL OR FUNDAMENTAL PECULIARITIES OF PROTOPLASMIC STRUCTURE IN RELATION TO THE CONDITIONS OF REACTIVITY

The structure of protoplasm is the subject of a special section in this textbook, and only those features which appear to be of special physiological significance, especially in relation to the problem of reactivity, need be considered here. The problem of what is essential or distinctive in the structure of living protoplasm—as distinguished from that of dead protoplasm or the simpler types of colloidal system—is a difficult one, largely because of the great diversity shown in the structural characters of different cells. In some cases the visible structural differentiation is slight, e.g., yeast cells, blood corpuscles, and even certain germ cells; in others it is highly complex, e.g., muscle cells, certain nerve cells, infusoria. Yet it seems clear that some basic or elementary structure must be common to all forms of protoplasm, in correspondence with those basic physiological properties and activities (reactivity, synthetic metabolism, and growth) which all have in common and which are known to be dependent on structure. The nature of this general or fundamental structure is not readily determined by direct observation; many forms of protoplasm, even those whose physiological behavior is complex, appear optically homogeneous under the microscope; an example is the clear protoplasm of the centrifuged egg cells described in Section IX, which nevertheless retain the power of development. The combination of observational and experimental (or physiological) methods of study seems the only possible means of obtaining insight into this problem; what is essential is that our conceptions of the structure of protoplasm should be consistent with—or help to explain—its fundamental physiological properties.

The present evidence indicates that the basic protoplasmic structure has a closer resemblance to an emulsion type of structure than to that of any other simple physical system. The essential feature in the structure of an emulsion is the presence of two (or more) fluid phases of different composition, one (or more) of which is in a fine state of subdivision and suspended or otherwise dispersed in the other, which is continuous. Fine subdivision implies a large surface of contact between the phases; hence the physical and chemical conditions at surfaces become of importance in determining the properties of the system. In this respect emulsions are similar to other colloidal systems.[1]

It is known that the structural stability of an emulsion system depends mainly on the conditions at the surface of separation between the phases; e.g., an emulsion of oil in water is not stable unless the surface layer has certain physical properties which prevent coalescence of the droplets. Both the electrical state of the transitional layer and its chemical composition are important. In most emulsions stability is secured by the presence of thin interfacial films of soap or other protective material. If these films are broken down the droplets reunite and the structure of the emulsion is destroyed; this occurs, for example, when strong acid (which decomposes the soap) is added to an emulsion of olive oil in alkaline water. This breaking down or "cracking" of an emulsion may occur under various conditions, chiefly mechanical and chemical, varying with the physical and chemical nature of the phases and of the stabilizing interfacial layer. What is of special interest from a physiological standpoint is that a relatively small quantity of a protective material, such as soap, may determine the stability of a large quantity of emulsion; it has been shown that the stabilizing surface film surrounding each droplet need not be more than a single molecule in thickness.[2] Correspondingly, a relatively slight chemical change may determine the breakdown of a large volume of emulsion. Thus the structural stability of an emulsion, like the structural stability of certain forms of protoplasm, may depend on

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[1] For a general account of emulsions, cf. Bancroft (1912–15); Clayton (1923).

[2] For the thickness and other properties of interfacial films, cf. Freundlich (1922),

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critical conditions which are readily altered, e.g., a certain range of H-ion concentration. In this respect there is an interesting analogy, which may be more than a superficial one, between the cracking of emulsions and the stimulation of highly irritable forms of protoplasm.

Living protoplasm appears to have the constitution of an emulsion in the respect that its structural stability, and hence many features of its chemical organization and behavior, depend on the presence of thin films (apparently consisting chiefly of lipoid material) by which its structural elements are inclosed and pervaded. The entire cell is inclosed by a thin, semi-permeable film, the plasma membrane; and there is evidence that in at least many types of cell the internal protoplasm is partitioned by films with similar diffusion-hindering properties; these films subdivide the cell into regions which are chemically and structurally dissimilar. A high degree of chemical differentiation within the limits of a single cell thus becomes possible; the basis for a stable and characteristic chemical organization is thus furnished. In correspondence with this structural differentiation a variety of physiological or metabolic activities may proceed side by side in the same cell without interfering with one another (cf. Hofmeister, 1901).

According to this conception living protoplasm is essentially a film-pervaded or film-partitioned system. The regions separated by these films and the films themselves are in many cases not optically distinguishable; but their presence at many protoplasmic boundaries—e.g., the general surface of blood corpuscles and other cells without distinguishable membranes—can be demonstrated by physiological methods, e.g., osmotic effects in anisotonic solutions. Inside the cell such boundaries are visible only when the adjoining regions differ in color, structure, or refractive index, e.g., in the case of vacuoles, nuclei, or alveoli; but the evidence from autolysis and other chemical phenomena in altered cells indicates their presence in many, perhaps all, cases.

An important difference between protoplasm and a typical emulsion like oil in water is that the two phases on the opposite sides of a protoplasmic film are not necessarily aqueous and non-aqueous respectively, but may both be aqueous. Any suspension of living cells in an aqueous medium illustrates this kind of condition, e.g., blood corpuscles in serum; both the interior of the corpuscle and the serum are complex aqueous solutions, and the film itself is too thin to be optically detectable as a separate structure. The boundary of the corpuscles is nevertheless distinct, and we can readily show by osmotic methods that a semi-permeable membrane is there present. Similarly within the limits of a single cell various structurally distinct regions, sometimes optically distinguishable, sometimes not, are to be regarded as bounded by thin films. This is presumably the case, for example, with the fibrillae of muscle cells, with neurofibrils, mitochondria, Golgi apparatus, and the single cilia in a compound structure like the ctenophore swimming plate. In many forms of protoplasm which appear homogeneous during life, the remnants of this film structure may become visible after death under certain conditions, e.g., in the reticular or honeycomb-like patterns of fixed and stained preparations.

As already mentioned, the presence of thin films with semi-permeable properties can readily be demonstrated in certain regions of the cell, e.g., at the external surface (plasma membranes), or at the surfaces of cell structures, such as nuclei, vacuoles, spheres, or alveoli. The chief physiologically important property of these films is their semi-permeability, or resistance to the diffusion of dissolved substances. It is well known that the semi-permeability of the plasma membranes disappears on death; semi-permeability thus appears to be a special feature of the living state; in all dead or dying cells the membranes become permeable to substances to which formerly they were difficultly permeable or impermeable. Conversely all permeability-increasing substances are destructive to living protoplasm (cytolytic substances). There is also evidence that this increase of permeability at death affects not only the most external protoplasmic film or plasma membrane, but also the film structure in the cell interior. The coagulative changes in dying cells, the changes then occurring in the physical consistency and tensile strength, and the phenomena of death rigor are evidence of profound structural changes in the protoplasm. Apparently the intracellular partitions undergo increase of permeability or breakdown at death; this is indicated by the fact that many chemical reactions which are absent or inappreciable during life proceed rapidly in dead cells; examples are the oxidase reactions which cause browning in fruits, potatoes, and leaves,[1] the production of large quantities of acid (like lactic acid), and the hydrolysis of proteins and glycogen in autolysis. All these reactions are promoted by cytolytic substances. The indications are that compounds which during life are kept apart by barriers of some kind become free to interact when the protoplasmic structure alters at death. Apparently the type of structure characteristic of living protoplasm is one by which free diffusion is prevented or restricted.

The chief facts of cell permeability are described in Section III, and its physical and chemical conditions are there discussed. Most of the existing observations relate to the permeability of the external protoplasmic layer or plasma membrane, but there is evidence that the intracellular membranes have similar properties; this is true also of the films formed in echinoderm eggs about injected droplets of salt solution (see Sec. V).

Of fundamental importance in relation to the problem of reactivity are the observations described in Section V showing the readiness with which the protoplasmic films may be broken down and replaced under various artificial and natural conditions. A normal instance of breakdown and re-formation of films is seen in the nuclear membranes during cell division. All such processes are the expression of chemical reactions of unknown nature; they indicate that an important part of the structure-forming metabolism of protoplasm relates to the formation and maintenance of film structure. Presumably in the formation of these films chemical reactions enter which are similar to those determining growth processes in general (synthetic reactions, including formation of specific proteins); these reactions typically require oxygen, but their special nature and conditions are still almost entirely unknown. The proteins of the surface films of blood corpuscles and other cells are specific, as shown by the existence of specific cytolysins. The protoplasmic film structure, once it is formed, appears to be the chief controlling factor in the interchange of diffusible materials between the different regions of the protoplasm and between the protoplasm and the exterior. The protoplasmic partitions, however, are not permanent or unchanging structures, but are to be regarded as varying in their continuity, permeability, and dependent properties (such as electrical polarization) according to the physiological state of the protoplasm and the external conditions acting upon it. These variations in film structure secondarily influence the rate and character of the chemical reactions in the protoplasmic system; evidence will shortly be presented indicating that variations of permeability are of constant occurrence in stimulation.

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[1] Harvey's recent observations on the leaves of the false indigo plant (*Baptisia*) are instructive in this regard (Harvey, 1921).

Since the formation of films is a characteristic feature of the processes at phase boundaries in polyphasic systems of all kinds, and especially in protoplasm (as the above-cited observations show), it is probable that films are present at all sharply defined boundary surfaces in the living cell, i.e., not only at the general surfaces of the cell and of cell structures like nuclei and vacuoles, but also at the surfaces of fibrils, granules, chromosomes, mitochondria, the Golgi apparatus, and other structures. Variations in the physical or chemical properties of these films will influence the catalytic and other properties of the surfaces, and hence the nature and rate of the chemical reactions occurring in the cell under surface influence. Electrical factors are of special importance in all such effects; this will be indicated more clearly in the sections dealing with electrical stimulation and bioelectric phenomena.

Surface conditions thus represent controlling factors of primary importance in the chemical reactions of protoplasm. Surface conditions also determine the catalytic activity of simple heterogeneous systems like the charcoal system, as already seen; this consideration explains why in its chemical behavior protoplasm exhibits so many features in common with such systems. An especially significant resemblance is the sensitivity of the protoplasmic reaction velocities to the presence of surface-active substances (narcotics).[1] These resemblances depend on simple general conditions common to all polyphasic systems. Protoplasm, however, differs from simple inorganic systems of this class not only in its far greater chemical complexity, but also in the possession of certain special features of structure and activity, and particularly in the high development of a system of variable surface films. Since these films are readily broken down and re-formed under often slight changes of condition, the state of the protoplasmic surface layers is subject to continual change; the rate and character of the chemical reactions occurring under the influence of these surfaces are affected correspondingly. There are many indications (apart from the action of narcotics) that the special sensitivity of the protoplasmic system to chemical, electrical, and other changes of condition, i.e., its irritability, is to be referred chiefly to this variability of the protoplasmic films. Direct evidence that many forms of stimulation and reaction are associated with changes in the permeability, electrical polarization, and other properties of the protoplasmic films or membranes will be presented below.

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[1] For the influence of surface-active compounds on the oxygen consumption of cells, cf. Warburg (1914, 1921).

III. GENERAL NATURE OF EXTERNAL CONDITIONS TO WHICH THE CELL REACTS

Any detailed description of the various conditions to which living cells and cell systems react is impossible in a single section, and we shall confine our discussion to the more general or fundamental conditions of this kind, using for illustration chiefly those cases where the phenomena are simplest and best known.

Responses to stimuli may be either simple or complex, but both may be called forth by equally simple changes of condition. Perhaps the most complex type of cellular reaction is that initiated in the egg cell by fertilization or artificial activation; the egg begins and under normal conditions carries through its long and complex cycle of development. This process is initiated in the starfish egg by a brief exposure to a solution of fatty acid, or in the frog's egg by simple puncture or the momentary passage of an electric current (see Sec. VIII). The comparatively simple reaction of a single irritable tissue (such as a muscle) is similarly elicited by slight mechanical or electrical changes. The general conditions of irritability and response have been most fully investigated in the isolated muscles and nerves of higher animals; but light is thrown on many characteristic features of cellular reactivity by the phenomena of fertilization and artificial parthenogenesis (see Sec. VIII). These phenomena also represent a response of the cell to changed conditions; perhaps their most conspicuous feature is the acceleration and characteristic modification of growth processes.

Growth and development depend essentially upon the formation and accumulation of protoplasmic structure through the metabolic reactions (specific syntheses) of the living protoplasm. What are known as "formative stimuli" and "formative response" are seen most clearly in these phenomena. A brief consideration of this type of reactivity is necessary here, since structure-forming reactions are universal in living protoplasm and probably play a part in all cellular activities, even in those which show little or no external evidence of structural change, such as the response of a nerve or muscle to stimulation (see below).

Generally speaking, in responses of the formative or morphogenetic type the synthetic metabolism of the cell is altered, in rate or character (or both), and the formation of structure is influenced correspondingly. The most striking illustrations are seen in fertilization and development. The egg cell begins a definite cycle of growth and structural transformation, in response to the change of condition introduced by fertilization or the artificial activating agent. Usually the process begins with a series of cell divisions; this is succeeded by the characteristic phenomena of differentiation and the formation of an embryo. In differentiation cells in different situations, and presumably exposed to different conditions, carry out different sequences of formative and physiological activity. These sequences represent in large part responses to stimuli. Although the conditions determining the precise nature of such responses are not understood in detail, it is recognized that two main groups of factors are concerned; these are the "external" and the "internal" factors of development. The internal factors are those contained within the cell itself, consisting in its special peculiarities of structure and chemical composition, or "organization," for example, the presence of a certain chromosomal complex; these factors fix more or less definitely the reactions of which a given cell or cell system is capable. Thus in the development of vertebrates the liver diverticulum gives rise only to liver cells, the cells of the neural plate to nerve cells, those of the germ tract to ova and spermatozoa, and so on. These various modes of formative activity are exhibited, however, only under certain definite external conditions; these are the external factors, which include temperature, oxygen supply, H-ion concentration, presence of specific chemical substances (hormones), mechanical conditions, light, gravity, etc. In many cases stimuli of a highly specific kind are necessary for the development of certain structures; e.g., in vertebrates the contact of the optic cup with the ectoderm induces lens formation (W. H. Lewis, 1904). Mechanical conditions are often highly important, especially in the growth of plant organs (e.g., tendrils), and electrical conditions undoubtedly play a highly important part as directive and correlating factors in many cases.[1]

The general conditions of stimulation, as exhibited more particularly in motor and other familiar types of reactivity, have been chiefly investigated in isolated tissues, especially muscle and nerve, which show a prompt and definite response to electrical and other stimuli. The general conclusions reached as a result of the extensive studies on these tissues are, however, widely applicable to other irritable systems, as the results of comparative physiology show. Apparently the primary process of excitation, i.e., the initiatory or releasing change occurring in the protoplasmic system (considered independently of the special nature of the response which follows), is essentially uniform in its physicochemical nature in all irritable forms of protoplasm. This is indicated by the universal susceptibility of irritable systems to the electric current and to mechanical stimulation; also by the readiness with which irritability is temporarily or reversibly abolished by surface-active substances (narcotics). Electrical sensitivity and narcotizability go hand in hand, and are apparently universal properties of protoplasm. Evidently these properties depend on fundamental features of structure or organization which all forms of protoplasm have in common. It is particularly to be noted that they both indicate a dependence of the primary processes of stimulation on surface conditions. Electrical polarizability, on which electric stimulation depends, and power of adsorption, on which narcosis by surface-active compounds depends, are properties of interfaces.

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[1] E.g., according to Kappers (1921) the direction of growth of nerve tracts in the central nervous system is controlled largely by electrical conditions; Ingvar's recent experiments support this idea (cf. Child, 1921, chaps. x, xi).

1. *Classes of stimuli:*

Stimuli are usually classed according to the physical nature of the stimulating agent as mechanical, electrical, thermal, radiant, chemical, osmotic, etc. Typically the stimulating condition is an event or external change of some kind, i.e., some variation in the incident conditions. The mere presence of a uniform physical condition is usually not sufficient for stimulation; the condition must change, and the change must be of more than a certain degree and rate. Thus an electric current may flow continually through a nerve without exciting it, but any sudden increase or decrease at once stimulates. Similarly with mechanical, radiant, or other stimuli: change of pressure or illumination is more effective than a constant condition. This rule may seem to have its exceptions, as in the stimulation of the retina by light of uniform intensity; but it is undoubtedly of wide application and physiologically significant. It should again be noted that the stimulating change of condition may result from the action of the organism itself rather than from any active change in the surroundings; this is seen, e.g., in the action of a heliotropic animal in a uniform light field; any accidental swerving to one side changes the illumination of the eyes or photoreceptors and calls forth a compensatory motor response; similarly with geotropic or other "tropic" responses.

2. *Selective irritability:*

Specialization in the sensitivity of cells or cell systems to different physical or chemical agents is a frequent phenomenon, especially in higher animals, and on it the selective character of many reactions depends. The irritable cell may be especially sensitive to light, changes of temperature, differences of osmotic pressure, differences of H-ion concentration, presence of special chemical substances, etc., while relatively insensitive to other stimuli. This condition is illustrated by the various sensory receptors of higher animals; these are distinguished as the thermoreceptors (temperature sense organs), chemoreceptors (in taste and olfactory organs), photoreceptors (in eye), kinetoreceptors (in tactile organs, equilibrium organs, the ear), according to *the physical nature of the agent to which they are specially responsive.* In each case the basis of this selective irritability is some special feature of structure or chemical composition, which heightens the sensitivity to the particular agent without as a rule interfering with the general sensitivity; thus the retina is stimulated by mechanical pressure or the electric current as well as by light of definite wave-lengths. In certain cases it is possible to determine the special feature on which the selective irritability depends; e.g., in light-receptor elements this appears to be usually the presence of compounds with special absorbent properties for rays of certain wave-lengths. The energy of these rays is thus more effectively transformed within the cell than would otherwise be the case, and the threshold of stimulation for light of these wave-lengths is lowered. Thus the curve showing the relative absorptive properties of the visual purple of the vertebrate retina for light of the visible wave-lengths corresponds closely to the curve of relative sensitivity to these wave-lengths (Hecht and Williams, 1921). Artificial photodynamic sensitization is apparently an illustration of the same phenomenon; many cells not ordinarily light-sensitive (*Paramoecia*, red blood corpuscles, eggs of marine animals) can be rendered so by adding appropriate light-absorbing compounds to their media. Eosin, for example, while indifferent to most cells in the dark, becomes strongly toxic in the light (Tappeiner, 1907). Irritable tissues (frogs' muscle and nerve) can be rendered photosensitive by staining in eosin and then placing in pure isotonic salt solutions, e.g., slightly alkaline Na acetate which heightens the general sensitivity; they are then stimulated by bright light from an electric lamp.[1] In an analogous manner specific chemical sensitivity is to be referred to the presence in the irritable cell of chemical compounds having some special affinity to the stimulating compounds. This is indicated most clearly by the phenomena of specific artificial sensitization or anaphylaxis. In the early stages of immunization of guinea pigs to any foreign protein the smooth muscle cells (e.g., in the bronchioles or uterus) become extremely sensitive to the protein in question and contract strongly in its presence while remaining indifferent to other proteins. In this case special compounds, "anti-bodies," situated apparently in the surface layer of the muscle cell, determine its special sensitivity to the antigenic protein.[2] In other cases of selective chemical sensitivity, as shown, e.g., in the olfactory sense, analogous conditions probably exist. Selective irritability to mechanical agents, so highly developed in special tactile organs, equilibrium organs, etc., depends on special features of mechanical construction (tactile hairs, otoliths, etc.).[1]

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[1] Unpublished observations in the Nela Research Laboratory, Cleveland.

[2] For a recent authoritative discussion of the mechanism of anaphylactic reaction, cf. Dale (1920).

3. *Types of response:*

The special reactions exhibited by the different organisms, tissues, and cells in response to stimulation of a single kind (e.g., electrical stimulation) are unlimited in their variety, and no general characterization is possible. Each kind of cell has its special peculiarities of action and reaction which depend on its inherited organization; e.g., a muscle cell contracts, a gland-cell secretes, a protozoön executes its characteristic motor reaction; and the nature of the response is not changed by changing the nature of the stimulus. In fact, in many irritable protoplasmic systems under normal conditions the response to any effective stimulus is not only qualitatively but quantitatively constant; either the cell does not respond at all, or it responds with full intensity; this type of response, the "all-or-none" type, is shown by the cardiac and voluntary muscle cells and nerve fibers of vertebrates and other higher animals, and by certain special cells such as nematocysts. It is especially characteristic of irritable elements with highly developed powers of transmission.[2]

Notwithstanding the diversification of the forms of response, and their independence (within limits) of the special nature of the stimuli, certain features of the stimulation reaction are remarkably constant and accompany all forms of stimulation, whatever the special nature of the response. These phenomena appear to be an index or expression of the fundamental physical and chemical changes which occur in all irritable forms of protoplasm during stimulation. Variations of electrical potential, changes of permeability, increase in oxygen consumption and carbon dioxide output, and a temporary loss or lowering of irritability (refractory period) are the chief. Typically these changes occur simultaneously or nearly so; their coincidence indicates that changes in both the chemical activity and the physical structure of the protoplasmic system are concerned in stimulation; they further indicate that the chemical and physical changes are interdependent. The nature of this interdependence will be considered below.

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[1] Sensitization to stimuli of various kinds, under the influence of special chemical compounds produced within the same organism, plays an important part in many physiological and developmental correlations. Cells may thus be rendered susceptible to growth-promoting or formative stimuli of a mechanical kind, as well as to stimuli of chemical or other kinds. For example, Leo Loeb has shown that in mammals the *corpora lutea* produce a specific substance which sensitizes the uterine mucosa in such a manner that incisions made in this structure induce a rapid cellular proliferation and the formation of deciduous tissue (*deciduomata*) (cf. L. Loeb, 1909, 1917, 1923). The normal growth of the placenta in relation to the implanted ovum depends on this reaction. Various other hormone effects, seen, e.g., in the growth of secondary sexual characters (horns, feathers, etc.), probably depend on conditions of a similar kind (cf. Marshall's review, 1923).

[2] For the "all-or-none" reaction of nerve, cf. Gotch (1902), Adrian (1914); of muscle, cf. Lucas (1909), Pratt and Eisenberger (1919).

One feature of normal stimulation processes which should be especially noted as of fundamental biochemical and physiological significance is their *reversibility*. Stimulation alters the chemical and physical state of the irritable system; evidence of this is seen in the fact that immediately after stimulation there is always a period, varying in length in different organisms and tissues, during which irritability is lost or greatly decreased. This period of complete or almost complete insensitivity is known as the "absolute refractory period"; it is succeeded by a somewhat longer period, "the relative refractory period," during which the properties of the system by degrees return to normal. Stimulation can then be repeated with the same result, as before. The duration of the whole refractory period varies in different tissues, from a few thousandths of a second (in nerve or voluntary muscle) to several seconds or even longer (in smooth muscle). The first or "absolute" part of the period evidently corresponds to the time during which the chemical and structural conditions necessary for stimulation are absent or deficient, apparently as a result of structural and chemical breakdown accompanying stimulation; these conditions are then restored (during the relative refractory period) by some process of chemical or structural reconstitution or repair. During this period processes are active which are apparently of the reverse kind to those associated with the excitation stage of the reaction; evidently the return to the resting state is an active process in which there is a removal or reversal of the conditions in which the irritable system finds itself as a result of stimulation. This reversal follows automatically upon the excitation effect proper; both must be regarded as forming part of a single cycle, consisting of alternate destructive and constructive stages.[1]

The case of the beating heart illustrates perhaps more clearly than any other the general nature of these conditions. Processes of chemical breakdown and energy production occupy the first or contractile phase of the cycle, and the system shows almost complete inexcitability during this period; during the second or relaxation phase, irritability returns progressively to normal; this phase represents the period of reversal, consisting essentially in reconstructive or reparative processes. The process of reversal is complete, as is shown by the fact that the system repeats the same cycle regularly for years on end. This case is typical; in all cases of reversible stimulation there is first some kind of disintegrative or destructive process which is succeeded by one of reconstruction. Claude Bernard[2] many years ago called attention to this characteristic condition; in any irritable system stimulation must automatically call forth or initiate a counter-process of repair if the system is to be a stable one. Hering (1897) has expressed a similar general conception of the stimulation cycle; he conceives of every stimulation as causing a chemical breakdown in the protoplasm (katabolic change) to which succeeds automatically a reconstructive process (anabolic change). This conception applies more particularly to those cases where the effects of stimulation are completely reversed, but this is not always the case; cells which break down on slight stimulation and show no recovery are well known; nematocysts and certain types of blood corpuscle (e.g., platelets and the explosive corpuscles of crustacea) are examples. In other cases cells which under normal conditions recover promptly and completely may show irreversible changes or breakdown as a result of excessive stimulation; this is well seen in the spinal cord of the strychninized frog, especially in lack of oxygen (Verworn, 1913, p. 170); even ordinary fatigue may be carried to a destructive point (cases of shock). It is important to note that the recovery from the effects of stimulation typically requires the presence of free oxygen, indicating that oxidations play an essential part in the reparative or synthetic processes by which the altered protoplasm is restored to its original state.

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[1] For a general account of the refractory period, cf. Tait (1910); Lucas (1912, 1917); Verworn (1913), chap. vii; also chap. xiv of my recent book on protoplasmic action (R. S. Lillie, 1923).

[2] *Leçons sur les phénomènes de la vie*, Vol. I, chap. iii.

Such evidence indicates that the recovery from stimulation is the result of processes which are different in kind and direction from those concerned in the initial or excitation stage. Recent studies of the refractory period of nerve and muscle lead to similar conclusions; the recovery process has apparently a higher temperature coefficient than the excitation process (Adrian, 1921), and is differently affected by lack of oxygen, cyanide, and various other poisons,[1] Certain special compounds, e.g., veratrin, interfere directly with the recovery of nerve and muscle from stimulation, while permitting the positive or excitation phase of the reaction; the veratrinized muscle contracts but fails to relax. The return phase of the bioelectric variation is also prevented (Tait, 1910). This latter effect is especially interesting since it indicates a failure in the repair of those structures, semi-permeable membranes, on which the normal bioelectric potentials of the resting cells depend. Evidence will shortly be presented indicating that the plasma membranes undergo temporary breakdown or increase of permeability during stimulation.

4. *Inhibition:*

Inhibition is decrease or arrest of physiological activity under the influence of some change of external or other condition; in this sense it represents the reverse of stimulation, and its underlying processes are probably closely related to those of the reversal phase of the normal stimulation cycle. During reversal the cell changes from a more to a less active state and the same is true of inhibition; in many cases there is also a decrease or loss of irritability, e.g., in the heart during vagus inhibition. Inhibition as a general physiological process is of equal biological importance with stimulation; this is seen in many reactions of the entire organism; e.g., cessation of muscular movement ("shamming death") is a favorite method of defense in all animal groups; and in most muscular reflexes the simultaneous inhibition and stimulation of opposing muscle groups take part (reciprocal inhibition).[1]

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[1] For further discussion and references to literature, cf. R. S. Lillie, *op. cit.* (1923), p. 341.

Inhibition may be characterized as a response of negative type, in which activity is decreased instead of increased. Many investigators have regarded it as an expression of the predominance of constructive or anabolic processes; these, being opposite or antagonistic to the katabolic processes underlying excitation, have the effect of interfering with or suppressing the latter. This point of view is a suggestive one, but is to be regarded as a guiding (or "first approximation") hypothesis rather than as a sufficient account of the conditions. It is evident that the problem of inhibition cannot be separated from that of stimulation; the same structures undergo change in the two cases, only the directions of the change are opposite. The most significant general fact is that in the action of the electric current on typical irritable tissues (muscle and nerve) excitatory effects and inhibitory effects appear under conditions indicating that they are physiological reciprocals; the current causes excitation, or induces heightened irritability, when it passes from cell to medium (at the cathodal electrode), and inhibition or depressed irritability when it passes in the reverse direction (at the anode). The general conditions of these effects will be discussed later under the subject of electrical stimulation.

It is noteworthy that certain types of innervation have an inhibitory effect; e.g., stimulation of the vagus arrests the heart and reduces the irritability of the cardiac muscle cells. An apparently related phenomenon is the inhibition of motor nerve cells in the central nervous system when the antagonist cells are stimulated (reciprocal inhibition of Sherrington). In many cases mechanical stimulation causes inhibition instead of excitation, e.g., in the defensive reflexes just cited; this is true also of some isolated irritable structures, e.g., the ctenophore swimming plate in sea water or artificial balanced media containing calcium; if calcium is removed the same treatment causes excitation (R. S. Lillie, 1906, 1908). The reversible arrest or decrease of activity or irritability under chemical influence (narcosis) is an effect which in many respects is closely allied to inhibition, and will be considered below.

IV. GENERAL NATURE OF CONDITIONS UNDERLYING THE RESPONSIVENESS TO STIMULATION

Since the energy of vital processes is in all cases transformed chemical energy, the chief effect of stimulation must be to alter the rate or character (or both) of the chemical reactions occurring in the irritable system. And since these reactions are controlled by the protoplasmic structure, stimulation must in some manner alter the structural conditions in the system. Further, the alteration in structure and in the dependent metabolic reactions is much greater than can be accounted for by the direct effect produced at the point of incidence of the stimulating agent. A pin prick may alter the behavior of the whole organism. This last consideration brings out another important general feature of all stimulation processes. The response is typically greater in extent, both in space and time, than the stimulus. There is a *transmission* of some effect, produced or initiated locally by the stimulating agent, throughout the whole responding system, i.e., a spread of physical or chemical influence of an activating kind. The entire response may also greatly exceed the stimulus in duration. These general features are characteristic of trigger effects or released effects, in which also the effect of the local change is transmitted to other parts of the reacting system and the total effect outlasts the releasing change.

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[1] Cf. Sherrington (1906).

A fundamental aspect of the problem of stimulation is thus indicated. The effect produced by the stimulus is one which has the property of propagating itself to a distance and involving other regions of the living system. The problem of transmission is thus inseparable from the problem of stimulation. Transmission of chemical effects to a distance in a system where chemical reactions are controlled by structure means transmission of structural change. In considering any typical case of stimulation, like that of a muscle cell or nerve fiber, we are thus led to examine into the general conditions under which structural change with dependent chemical change can be transmitted to a distance.

In the typical stimulation, e.g., of a nerve, there is a *local* effect, produced by the stimulating agent at its point of incidence, and a *propagated* effect, consisting in a chemical and structural alteration which travels from the point of stimulation over the whole irritable element. The local effect may vary in its physical character; e.g., a sudden pressure, a change of temperature, an electrical or osmotic change, may alter the nerve locally and stimulate; the propagated disturbance which is thus initiated has a constant and definite character which is dependent on the special inherited constitution of the system. In a frog's nerve at 20° the wave of excitation travels at a rate of 30 m. per second; the wave is accompanied by certain physical changes, especially a variation of electrical potential; when it reaches another irritable system with which the nerve is connected, it arouses the characteristic reaction of the latter, e.g., causes muscular contraction, or secretion, calls forth a reflex action, changes the state of consciousness, etc.

It is, therefore, not the direct effect produced by the stimulus but the indirect or transmitted effect which causes the characteristic change of physiological activity or response. In a muscle cell the transmitted disturbance is not identical with the contraction, but merely furnishes the condition under which the contractile mechanism is activated. It is possible to dissociate transmission from contraction, e.g., by partly immersing the muscle in distilled water for a sufficient time; the swollen or water-logged portion of the muscle will then transmit excitation to the unaltered still contractile part, although itself incapable of contracting (Biedermann, 1888).

In order, therefore, that any irritable system shall react as a whole the exciting condition must be transmitted to all of its parts. The problem of stimulation thus resolves itself largely into the problem of the physicochemical nature of the propagated disturbance. A feature of the excitatory influence in most organisms is that it travels along definite, structurally defined paths. This is best seen in the nervous system of higher animals; but even within single cells there is evidence of such conducting paths, e.g., in the intracellular strands of Protozoa (Taylor, 1920) and the neurofibrils of nerve cells. Physical continuity of a definite kind of structure is thus a requirement for the protoplasmic type of transmission; and the transmission is associated with chemical change as well as with a change of structure. Certain types of transmission in inorganic systems exhibit similar general characteristics, e.g., the transmission of combustion along a fuse, and transmissions dependent on local electrochemical action, e.g., the transmission of activation in passive metals. Transmission along a fuse depends on local rise of temperature, of which there is no evidence in nerve, so that the analogy of combustion waves or explosion waves seems excluded. On the other hand, the analogy with the type of transmission seen in passive iron in nitric acid, and in similar systems in which local electrical circuits form the condition for propagation, appears to be a close one, since there are many indications that in nerve and other living systems the electrical variation, which is always associated with the local excitation, is the essential condition in which the transmission depends.

Transmission of chemical influence to a distance through the intermediary of electrical currents is a well-known phenomenon in inorganic systems, seen, e.g., whenever a battery is connected with an electrolytic cell. When the circuit is closed, chemical change begins simultaneously in both battery and cell, and the rate of change in the one system is controlled by that in the other in accordance with Faraday's law. The phenomena described by Ostwald (1890) under the name of "chemical distance action" are of similar nature; the passage of a current through a circuit involves chemical change at the surface of the electrodes, i.e., wherever the current passes between the metallic and the electrolytic phases of the system. The possibility that the transmission of physiological influence (excitation or inhibition) to a distance in protoplasmic systems—what may be called "physiological distance action"—is a special case of chemical distance action thus requires consideration. The above-described structural peculiarity of protoplasm, sharp demarcation between protoplasm and medium or between the protoplasmic phases, is comparable in a general physical sense with the sharp demarcation between electrode and electrolyte solution in an electrolytic cell. In both cases similar physical effects (polarization) occur when a current is passed across the boundary. At the surface of an electrode, this polarization, if sufficient in degree, is associated with chemical change; there is also evidence of chemical effects when a strong current is passed between two electrolyte solutions separated by a thin film or membrane of high electrical resistance, a condition favorable to maximal polarization (Lillie and Pond, 1923). It is therefore to be expected on general physical grounds that chemical effects will result when a current is passed through the living protoplasmic system, since in this case also the semi-permeable surface films are the seat of polarization.

The general view that electrical factors—specifically, the passage of bioelectric currents between the different regions of the protoplasmic system—are concerned in the rapid transmission of physiological influence in nerve and other conducting elements is also suggested by the general susceptibility of living cells to the electric current. Electrical sensitivity appears to be a universal property of protoplasm. The sensitivity of nerve, muscle, and other highly irritable protoplasmic systems to the electric current has been known since Galvani's time; irritable structures in plants (e.g., in sensitive plants) are also electrically sensitive; Protozoa show definitely oriented swimming movements in the electric field (electrotaxis); and the directive influence of electric currents on growth (galvanotropism) has recently been demonstrated with great clearness in animals (Lund, 1921). Apparently growth, the fundamental vital process, may be promoted or inhibited—i.e., its rate altered reversibly in either direction—by the electric current.[1] It is also known that variations of bioelectric potential accompany all cases of stimulation; i.e., the living system in its own activity gives rise to electric currents which traverse the protoplasm and the surroundings. These various facts favor the general hypothesis that in all cases of stimulation and transmission the electrical factor is the controlling one. This view implies that stimulating agencies other than the electric current produce their effects in an indirect manner by altering the electrical conditions in the living system. If the electrical factor is the primary one, transmission of physiological influence to a distance becomes in a general sense intelligible on the basis of the physicochemical constitution of the protoplasmic system. Protoplasm and the cell media contain salts and are good electrolytic conductors; and the characteristic structure, aqueous phases separated by thin, alterable, semi-permeable membranes, furnishes the conditions for differences of potential, and hence for the production of electric currents.

It has often been pointed out that the living system, considered from a simple physicochemical point of view, corresponds to an electrolyte solution partitioned by thin semi-permeable membranes. As an electrolyte solution it is a good conductor of electricity; the semi-permeable membranes interpose resistance to the diffusion of ions and furnish the conditions for polarization, i.e., for setting up differences of electrical potential between the opposite faces of the membranes. In a system of this structure both physical and chemical effects may be produced by passing a current, since polarization effects, if sufficient, may furnish the conditions for chemical changes which secondarily may cause structural changes in the membranes with resultant variations of permeability and polarization.

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[1] Cf. my recent article "Growth in living and non-living systems" (R. S. Lillie, 1922).

We may now ask the question, why should the passage of an electric current through a living tissue for a brief period cause stimulation? The answer to such a question is probably the same, from a general physical point of view, as the answer to the question why the passage of a current between a passive iron wire and the surrounding solution of nitric acid should cause activation. Physically, what happens in the latter class is to be conceived essentially as follows. The passive metal, in the absence of disturbing conditions, is protected from reaction by the presence of a thin impermeable film of oxide. When the metal and the solution are connected to a battery or other source of current a state of electrical tension (P.D. or polarization) is set up across the boundary; when this tension is sufficient, electricity (electrons) passes between the molecules of the metal and those of the solution; this (when the metal is cathode) involves the reduction of the passivating film to a lower state of oxidation and its consequent breakdown. Chemical reaction between metal and solution is thus initiated and proceeds. The initiation of the reaction depends on the removal by electrochemical action of a protecting film impermeable to the acid. The automatic transmission of a wave of activation along a passive wire (to be described below) depends on similar electrochemical effects produced by the local currents between the active and the passive areas.[1] The initiation of an extensive chemical reaction by a brief local physical or chemical alteration is the essential feature in which this phenomenon resembles the response of an irritable living system to stimulation. The living system, e.g., nerve axone, has certain structural characters in common with the passive iron system; in both there is a thin chemically alterable film interposed between two conductors. In the living system also the presumption is that the stimulating current acts by causing a polarization at the boundary, with resultant chemical effects which alter the film; the local bioelectric currents then furnish the conditions for the spread of the effect. This parallel will be discussed more fully below.

Connection between surface changes and stimulation:

It is important to note that in the inorganic systems (battery or electrolytic cell) in which the passage of a current induces chemical change the reaction occurs only at the surface of contact between the electrolyte solution and the electrode, not in the interior of the metallic or of the electrolytic phase. Electrochemical reactions are surface reactions. This general fact is to be correlated with the general physiological observation that the critical or initiatory changes in stimulation originate at the surface of the irritable element, i.e., at the boundary between the cell or cell structure and the surrounding medium. Surface reactions are those concerned in the initiation of stimulation and apparently also in its spread. A brief résumé of the chief facts indicating this relationship of surface changes to stimulation may now be added.

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[1] For an account of the phenomena of passivity in metals, cf. the recent review of Bennett and Burnham (1917).

An especially significant fact is the sensitivity of many cells to slight mechanical contact; this is seen in a great variety of organisms, and indicates that the physical effect releasing the propagated disturbance need affect directly only the cell surface. Blood corpuscles and other cells frequently break down on the contact of capillary needles; contact sensitivity of all kinds illustrates the same condition. Chemical stimulation and chemical alteration of irritability (anaesthesia, sensitization) appear also to be surface effects in most cases; many chemical compounds produce their physiological effects without evidence of penetration into the cell interior; examples are the stimulation and sensitization of muscle and nerve by isotonic salt solutions (Ca precipitants, etc.), anaesthetization by Mg salts and isotonic sugar solution; increase of oxygen consumption in sea-urchin eggs by non-penetrating alkalies (NaOH, KOH). In the case of electrical stimulation the quantitative determinations of Nernst, Lapicque, Lucas, and others, showing the parallelism between the stimulating action of the current and its polarizing action, also indicate that stimulation originates at the polarizable surfaces of the cell, i.e., the semipermeable boundary layers or membranes. That this is the case is also indicated by many characteristic structural conditions in the nervous system of higher animals and in other regions where stimuli are transmitted from one cell or irritable element to another. Nerve end plates spread over the surface of the muscle cell, effecting intimate contact but not penetrating; nerve cells in the central nervous system are related to one another by means of end feet, interlacing terminals, or similar structures which establish contact without penetration or protoplasmic fusion. From these various facts we must conclude that in irritable cells the plasma membrane is not merely a protective layer but plays the part of a sensitive intermediary between the external world and the internal protoplasm.[1]

The indications are that any slight local physical change at the cell surface, causing stimulation of the cell, does so by altering the electrical conditions at that area, thus initiating an electrically transmitted disturbance. This view implies that all forms of stimulation are electrical; i.e., the primary stimulating agent produces its effect by changing locally the electric potential at the cell surface. The local bioelectric current then arising between the altered region and the region adjoining is the first step in the stimulation proper. Local bioelectric currents are known to be produced in muscle and nerve by many kinds of alteration, mechanical, chemical, or other, the altered region becoming negative relatively to the adjacent unaltered region; a current (current of injury, demarcation current) then flows between the two regions, and this current can be shown to be of sufficient intensity to cause stimulation. As we shall see later, the evidence indicates that transmission is a result of the secondary stimulating action of local bioelectric currents. The conditions of electrical stimulation are thus of fundamental importance and will now be considered in some detail.

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[1] For a fuller account of these various conditions and references to the literature, cf. R. S. Lillie (1922); also R. S. Lillie (1923), chap. xiv.

V. GENERAL CONDITIONS OF ELECTRICAL STIMULATION

Not only the passage of an electrical current from some outside source through an irritable tissue, but other changes in its electrical condition may cause stimulation, e.g., the passage of a spark between the terminals of an electric machine near a nerve held parallel to the discharge. In this case the stimulating effect depends on induction,[1] but the conditions are not essentially different from those present when a battery current is passed lengthwise through the nerve. What is common to both cases is the movement of ions in the irritable element or its surroundings; this implies that the essential change in stimulation is the displacement of ions relatively to the structural surfaces which control the chemical reactions of the protoplasm.

Highly irritable tissues like nerve are very sensitive to sudden changes in electrical conditions, although a stationary condition, e.g., that accompanying a constant flow of current through the nerve, does not stimulate. Yet, although a steady current does not stimulate during its flow, it changes locally the state of the tissue so as to render the latter more or less irritable than it is while the current is not flowing (electrotonus).

The most generally significant features of the electrical stimulation of irritable tissues are (1) that it occurs at times of *change* of current (make or break, increase or decrease), (2) that the change of current must be at more than a certain *rate*, and (3) that the stimulating effect has a *polar* character, i.e., depends on the direction of the current relatively to the protoplasmic surfaces. The chief relations between the variables of an electric current and its stimulating action will now be briefly considered under the headings: intensity, duration, rate of change, direction (polar character), intermittence (summation effects).

1. *Intensity:*

An electric current passed through an irritable tissue must have a certain minimal or threshold intensity in order to stimulate. This intensity varies greatly for different tissues and for the same tissue under different conditions (fatigue, narcosis, temperature, chemical composition of medium, etc.). A current of one millionth ampere or even less passing along a sensitive frog's nerve through platinum electrodes 2 cm. apart is sufficient to stimulate on either make or break. The current required to activate starfish eggs is many hundred times more intense. Such comparisons are difficult to make precisely, because the exact proportionality between the total current traversing a tissue or suspension of cells and the fraction which actually effects the stimulation cannot be determined. The local intensity of the current in the different regions of a structurally and chemically complex conductor like a living tissue is determined by the local conditions of resistance, as well as by other factors of a physiological kind, e.g., the presence of polarization effects, inductance, or local bioelectric currents. It is known that in a suspension of living cells, e.g., blood, the cells play little part in the total conduction (cf. Höber, 1914, p. 372). In comparing the sensitivities of different irritable systems to the current, the most satisfactory method is to determine the *density* of current required to produce the physiological effect, i.e., the current (in milliamperes) traversing a unit sectional area (e.g., square millimeter); this value is obtained by dividing the total current by the sectional area of the conducting path though tissue and medium. A current of less than 1δ (δ=.001 milliampere per square millimeter) lasting a few thousandths of a second will stimulate a frog's sciatic nerve; voluntary muscle requires a density several times higher; *Paramoecia* show their optimum galvanotropic response at *ca.* 20δ (cf. Davenport, 1908, p. 142); to promote the growth of a hydranth from the anodal surface of a cut stem of the hydroid *Obelia* a current of 30 to 50δ is required (Lund, 1921); while to activate the starfish egg, the required densities are much higher.[1]

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[1] Cf. J. Loeb (1906), p. 99.

2. *Duration:*

An electric current, of threshold or higher intensity, must flow continuously through the irritable tissue for more than a certain minimal time in order to stimulate. This relation between duration of flow and stimulating effect is highly characteristic; it shows that the current causes some progressive change in the irritable system as it flows, and that it is only when this change has reached a certain critical stage that the stimulation reaction is initiated. The phenomena of summation (p. 199) also show the importance of the duration factor in electrical stimulation. The determination of the least duration required by the stimulating current of threshold intensity in the case of any irritable tissue is highly important in the physiological characterization of that tissue, since this time is an index of its characteristic rate of reaction or specific time factor of stimulation (chronaxie). Different irritable structures vary widely in this respect; in certain highly irritable elements (nerves, motor end plates) a duration of a few thousandths of a second is sufficient, while sluggish or slow tissues, e.g., smooth muscle, require several seconds. Lucas (1910) gives the following times for the tissues of the frog; substance β of the sartorius (nerve end plate), .001 second; motor nerve trunk, .003 second; voluntary muscle cell (sartorius), .02 second; ventricular muscle, 2 seconds. Lapicque (1905) has made a comparative study of the time factor in the muscles of various vertebrate and invertebrate animals; in general this factor is closely correlated with the characteristic rate of response of the tissue, i.e., speed of contraction in muscle or of transmission in nerve. Rapidly reacting elements are stimulated by currents of brief duration and vice versa. It is especially to be noted that such elements have brief and rapidly changing bioelectric variations, a highly significant correlation. The term chronaxie was introduced by Lapicque (1909) to designate the characteristic time factor of an irritable tissue; it is at present defined as the briefest effective duration of the stimulating current of twice the threshold intensity. Chronaxie appears to be essentially an expression of the characteristic rate at which the chemical reactions determining excitation proceed in the irritable elements; it is constant for a given tissue under normal conditions and at a definite temperature; yet it is readily altered (usually prolonged) by conditions (fatigue, changes in composition of medium) which influence sensitivity (Lucas, 1908, 1910).

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[1] Unpublished observations at the Marine Biological Laboratory, Woods Hole.

TABLE I*

SARTORIUS OF TOAD

T 10.2°

t Duration of Current in Seconds	$\sqrt{t} \times 10^6$	i Threshold Current (Volts)	$i\sqrt{t}$
0.014	118	0.45	53.1
0.010	100	0.45	45.0
0.007	84	0.46	38.6
0.0052	72	0.46	33.1
0.0035	59	0.48	28.3
0.0017	41	0.52	21.3
0.00087	29	0.69	20.0
0.00052	23	0.83	19.1
0.00035	19	0.99	18.9
0.00017	13	1.46	19.0

* From Lucas, *J. Physiol.*, 36, 113, 1907.

As the intensity of a stimulating current increases above the threshold, the necessary time of flow decreases, although at a slower rate. An approximation to a relation in which the product of the intensity into the square root of the minimal effective duration is constant is highly characteristic. This relation has been interpreted by Nernst (1908) as indicating that the critical (releasing or initiatory) change in stimulation is a change of polarization at the semi-permeable membranes of the irritable tissue. The same relation can be mathematically derived on the assumption that the essential effect of the current is to transport ions to the semi-permeable surfaces (Nernst, 1908); it has also been shown by Lapicque (1901) to hold for the production of polarization currents from dead partitions. It seems thus certain that the current stimulates by means of its polarizing action at the protoplasmic films or partitions; apparently the primary effect of the current is produced at the external film or plasma membrane.

The typical relations between intensities and minimal durations in stimulating currents are well illustrated by Table I which summarizes a series of observations by Lucas on voluntary muscle (Lucas, 1907). The tissue (toad's sartorius) was stimulated in its nerve-free region by currents of measured duration—the motion of a pendulum making and breaking the circuit through the tissue. It will be noted that the threshold intensity (proportional to the voltage) undergoes little change until the duration of current is shortened to .0017 second. As the duration is decreased beyond this point the voltage required for stimulation increases; through a considerable range of durations (down to 0.00017 second) the product $i\sqrt{t}$ remains nearly constant.

Recently the electrical stimulation of single muscle cells has been investigated by Jinnaka and Azuma (1922), using as stimulating (cathodal) electrode a capillary tube filled with Ringer's solution and perforated by a hole $10\,\mu$ in diameter applied to the muscle fiber (frog's sartorius). The time interval between make and break was measured by adjusting the distance between two contacts on a graduated circular disk rotated by a spring; the measurements were controlled by a string galvanometer and tuning fork. The threshold intensity for the single cell was found to be one or two microamperes (millionths of an ampere). Table II gives a series of their observations. The required intensity begins to increase at a duration of about .001 second, and, while the product $i\sqrt{t}$ fluctuates considerably through the range down to .0001 second, the deviation is not marked until the last observation. The single irritable cell thus exhibits the same kind of behavior as the entire muscle.

TABLE II

ELECTRICAL STIMULATION OF A SINGLE MUSCLE CELL
(JINNAKA AND AZUMA)

t Duration of Current in Seconds	i Intensity Required (Microamperes)	$\sqrt{t\times 10^8}$	$i\sqrt{t}$
∞	1.545	....	....
.002	1.545	447	691
.00181	1.548	425	658
.00162	1.551	402	623
.00143	1.553	378	587
.00124	1.557	352	548
.00105	1.565	324	507
.00086	1.581	293	463
.00077	1.627	277	450
.00057	1.812	239	433
.00038	2.442	195	476
.00019	3.94	138	544
.00010	7.229	100	723

The square root relation is also shown by nerve and by sensory elements. Nernst (1908) has shown that the same rule holds for alternating currents; the stimulating effect is an inverse function of the root of the frequency (m); i.e., for a constant degree of stimulation $i/\sqrt{m}$ is approximately constant. Hence rapidly alternating currents of high intensity may be passed through the human body without apparent effect. Evidently in order to be effective the current must flow in one direction for a sufficient time, presumably the time required to effect a critical change of polarization.

The square root rule is valid only within a certain range of durations and intensities; thus a current of less than threshold intensity cannot be made to stimulate by simply prolonging its duration. Hill (1910), Lapicque (1908), and others have endeavored to reach a more exact and comprehensive formulation of the relations between intensity and duration, taking into account the characteristic structural and other differences between the living irritable element and the simple polarizable partition considered by Nernst. These modifications of the simple polarization theory cannot here be considered in detail. Hill points out that the two membranes on the opposite faces of the cell are both concerned in the polarization, and that their distance apart must be considered.[1] The special metabolic rate of the cell is also a factor determining the rate and degree of polarization change produced by a given current; i.e., special physiological conditions are co-present with (or superposed upon) the simple physical conditions; this is shown perhaps most clearly by the phenomena of stimulation by changing currents.

3. *Rate of change:*

It is well known that if a current too weak to stimulate be passed through a nerve or muscle and then gradually increased, it may attain a high intensity (compared with that required to stimulate with sudden closure) without stimulating; similarly a gradual decrease of an already flowing current is ineffective, while a sudden decrease or break will stimulate. Evidently what is required for stimulation is not simply that a current of sufficient intensity should flow through the tissue, but that the intensity should change at more than a certain rate. The rate of change (di/dt), or intensity-time gradient, required for stimulation can be determined with exactitude by special methods; e.g., in Lucas' investigation cited below the current was varied by means of a sliding shutter which opened or closed a rectangular opening or slot in a partition set across a bath of $ZnSO_4$ solution forming part of the stimulating circuit. By varying the rate of movement of the shutter the intensity of the current could be increased or decreased at any desired rate.

On comparing the stimulating effects of uniformly increasing currents of different rates of change on such a tissue as nerve, it is found that a certain minimal rate of change is required in order to stimulate. This rate of change is rapid for nerve, slower for voluntary muscle and slowest for cardiac and involuntary muscle; i.e., the order is the same as that of the respective chronaxies. With a rate of change rapid enough for stimulation the current must be increased to a certain intensity before stimulation results; this intensity is lower the more rapid the rate of increase. For example, Lucas (1908) found the following values (Table III) for a nerve which was stimulated by a current of 5 microamperes with instant closure.

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[1] This consideration explains why a current traversing a nerve or muscle at right angles to its length is comparatively ineffective. The surfaces at which the current lines intersect the opposite faces of the irritable element are then too close together and the oppositely oriented polarization effects neutralize each other.

TABLE III

Time Required to Reach Stimulating Intensity (Seconds)	Intensity Required (Microamperes)
0	5
.031	7
.051	11.5
.09	18.5

Slowly increasing currents must thus reach a higher intensity than rapidly increasing currents in order to stimulate; if the rate of increase is too slow even strong currents fail to stimulate. A minimal intensity gradient may thus be determined which is characteristic of the tissue; this gradient varies with temperature (within the physiological range of 5°–35°), being steeper for high temperatures.

On the polarization theory of stimulation these results indicate that the electrical state of the irritable element (i.e., its polarization) must be altered at more than a certain rate in order to initiate the stimulation reaction. Apparently the cell has the power of compensating for externally induced changes of polarization provided these changes are not too rapid. The indications are that a certain normal or physiological state of surface polarization, corresponding to the resting state, tends automatically to be maintained by the regulative activity of the cell. To disturb this polarization to a degree sufficient for stimulation the stimulating agent must act at a more rapid rate than can be compensated by this regulative activity. The return phase of the bioelectric variation, which is the index or expression of returning polarization, is in fact rapid for rapidly responding tissues and vice versa. The special relations of the bioelectric variation to stimulation will be considered below.

4. *Direction:*

It is characteristic that the current exerts its immediate stimulating or other action only where it enters or leaves the irritable tissue, also that the physiological effects produced at the regions of entrance and exit are opposite in kind; i.e., the action of the current is typically *polar*. This is perhaps the most significant feature of electrical stimulation from a physicochemical point of view, for it suggests directly a comparison with the conditions in a battery or electrolytic cell, where a similar reciprocality of action at the two electrodes is seen. What determines the character of the chemical effect at either electrode is the direction of the current between electrode and solution. Where the current (positive stream) enters the solution from the metal (i.e., at anode) it produces chemical effects which in general are of an oxidizing kind; where it leaves the solution (at cathode) the effects are reducing. In an analogous manner, when electrodes are applied to a typical irritable tissue (nerve or muscle) and the current is made, it can be shown that stimulation originates at the region adjoining the cathode, i.e., where the current passes from the living tissue to the surroundings; at the other electrode the effects are of the physiologically reciprocal or inhibitory kind. Conversely, when the already flowing current is broken, stimulation originates at the anode, and there is momentary inhibition at the cathode; these effects are apparently the results of a current of reversed direction, the polarization current, which flows momentarily through the tissue when the stimulating current is broken. This relation of the stimulating effect of the current to its direction of flow is known as the law of polar stimulation.

These experiments show that the direction of the current, relatively to the cell surface, determines the nature of its physiological effect. Since the current stimulates by its polarizing action, they show further that the physiological effect produced by a decrease of polarization is of the reverse kind to that produced by increase. The direction, as well as the rate and degree, of the change of polarization is of critical importance. Since the evidence from the bioelectric phenomena indicates that the surface of the resting cell is the seat of a potential difference or physiological polarization of constant orientation, positive externally, negative internally, it is clear that an external current stimulates only when it passes in such a direction as to decrease this polarization. Electrical stimulation, therefore, corresponds to *depolarization*, which must be sufficient in rate and degree, electrical inhibition (in anelectrotonus) to *increased polarization*, i.e., reinforcement of the already existing polarization.

Polarity in the physiological action of the electric current is seen in many other effects, e.g., polar disintegration of cells (in Protozoa, sea-urchin eggs), electrotaxis, influence of current on growth and regeneration, influence on sensitivity (electrotonus).

5. *Summation:*

A single brief mechanical or electrical stimulus may have no apparent effect upon an irritable cell or tissue, while two or more such stimuli in sufficiently rapid succession will stimulate. This is the phenomenon called summation; evidently the effect produced at the end of a succession of subminimal stimuli is an additive one; each member of the series produces some latent effect, but the physiological response does not appear until the end of the series is reached.

In all summation phenomena the time factor is of primary importance. We have seen that too brief closure of a current of threshold intensity will not excite a nerve or muscle; a succession of two or more such brief currents will also have no effect unless they follow one another by less than a certain interval.

This interval is called the "summation interval"; its duration varies for different tissues, and has a definite relation to the characteristic chronaxie, being brief for rapidly reacting tissues and vice versa (Lucas, 1910). In general the interval is shorter than the minimal duration of the current of threshold intensity. Lucas (1910) gives the following measurements for the summation intervals of three irritable tissues of the frog at 13°:

Motor nerve (sciatic)	.0004–.0005	second
Voluntary muscle (sartorius),	.0011–.0019	second
Heart muscle (ventricle)	.008	second

In each experiment two induction shocks were used which were 5 per cent below the strength required for effective single stimuli; when the shocks were 10 per cent below the threshold the interval was much shorter. Evidently the first shock, while not exciting the tissue, puts it temporarily into a state where a second shock will excite. This state of increased excitability disappears spontaneously in a brief time; this time is shorter the more rapid the excitation process (i.e., the briefer the chronaxie); if before this time has elapsed the tissue is again excited it responds. The local change produced by the single subminimal stimulus is not propagated; the summation effect shows, therefore, that the local effect must attain a certain level in order to produce a propagated or transmitted disturbance which activates the whole irritable element. The distinction between "local change" and "propagated change," as well as the relation between the two, is seen perhaps most clearly in the summation effect. The temperature coefficient of the summation interval is of the same order as that of diffusion ($Q_{10} = ca.\ 1.3$) (Lucas, 1910), a fact indicating that the local change is one of a purely physical kind not involving metabolism—presumably a polarization change.

The general theory of the change produced in the irritable tissue or cell by the electric current may be briefly summarized as follows. The essential physical effect caused by the current is a change of polarization at the semi-permeable membranes of the irritable element. Hermann and other physiologists had also inferred that the stimulating effect of the current was connected with its polarizing action. They had observed that a counter-electromotive force (polarization E.M.F.) instantly arises in a tissue through which a current is passed. Hermann further concluded from the law of polar excitation that a negative polarization (one making the cell surface more negative) corresponded to excitation, and a positive polarization to inhibition. Later Nernst modified and extended the polarization theory to satisfy the requirements of modern physicochemical conceptions. Ions are moved by the passage of the current, and are blocked at the semi-permeable partitions or membranes of the tissue; this effect is opposed by back-diffusion along the gradients thus set up. A certain time is required to change the concentration of ions at the semi-permeable surfaces. Assuming that the essential factors are the transport of ions to the membrane by the current and the opposing forces of diffusion, the degree of polarization produced should be proportional to the intensity of the current and the root of its time of flow ($P = K i\sqrt{t}$). When the polarization reaches a certain degree, the irritable cell is in some manner aroused to activity, or stimulated. The rule that the stimulation effect varies with the polarization effect holds true for many tissues, as we have seen, but as a rule only under certain limitations. It seems clear, however, that the electric current stimulates primarily because it polarizes. If the polarizing effect is in the direction of decreasing the already existing or physiological polarization, and is sufficient in degree and rate, stimulation results; polarization in the reverse sense counteracts or opposes stimulation, i.e., produces inhibition. The fact that the polarization change must occur at more than a certain rate indicates that the cell tends to maintain a certain state of polarization, corresponding to the resting condition, and automatically compensates for disturbances of polarization if these are not too rapid.

VI. CHANGES ACCOMPANYING THE RESPONSE OF THE IRRITABLE SYSTEM TO STIMULATION

Certain definite physical and chemical changes in the irritable system appear to accompany all forms of stimulation and activation; of these the chief are: (1) variations of electrical potential (bioelectric variations); (2) changes of permeability; (3) temporary loss of excitability (refractory period); (4) chemical or metabolic changes; (5) heat production. Of these the first three are of especial significance; they are constantly associated and appear to have similar time relations; all of the evidence indicates that they depend on the same fundamental condition, namely, a temporary alteration or breakdown of semi-permeable protoplasmic partitions, especially the external surface film of the cell, or plasma membrane. Increased oxygen consumption and output of CO_2, and increased heat production are also general effects of stimulation; these effects vary greatly in different cells and tissues, according to the special nature of the response. It is significant that in the simplest irritable tissue, nerve, where excitation and transmission are dissociated from response of a special kind, the chemical and thermal effects of stimulation are so slight that their demonstration is difficult or uncertain. This fact indicates that the transmitted excitation effect which releases the response involves little chemical or energetic change on its own account. The increase in metabolism and heat production is greatest in those tissues where stimulation leads to a large expenditure of energy, as in muscle. These effects, however, are to be regarded as secondary rather than primary effects of stimulation; i.e., in these cells stimulation initiates or releases extensive chemical and physical changes, but the inciting process itself, the transmitted wave of excitation, represents only a small portion of the total effect.

These several manifestations of stimulation will now be briefly reviewed in order.

1. *Bioelectric variations:*[1]

Variations of electrical potential accompany all forms of response to stimulation, apparently without exception. As already stated, they are associated with variations of permeability and sensitivity, and are characteristically reversible. The direction of the variation of potential accompanying increased activity appears to be constant, in the direction of increased negativity; i.e., any increase in physiological activity, of whatever kind (growth, contraction, secretion, nervous activity), is associated with a negative variation. The reversed electrical change, in the direction of greater positivity, is typically associated with a change from a state of greater to one of less physiological activity, e.g., with a return of the reacting system to or toward the resting state, or with processes of inhibition. The two phases of the simple bioelectric cycle thus appear to be the expression of processes of opposite kind occurring in the protoplasmic system.

Typically in any irritable tissue the bioelectric variation is the first evidence of response; in vertebrate muscle it precedes the mechanical response or contraction; in the case of a single twitch it may even be completed before contraction has begun (cf. Snyder, 1913). The electric variation is thus evidence of some physical change in the cell preceding and determining the response; there is no evidence of the existence of a response unaccompanied by an electric variation. A further significant fact is that the time relations of the bioelectric variation are closely correlated with those of the refractory period; apparently the first or rising phase of the variation corresponds closely in its duration with the absolute refractory period. The indications are that both phenomena are expressions of the same physical change in the protoplasmic system, viz., a structural change in the plasma membrane involving a temporary breakdown or increase of permeability. The inexcitability during the rise of the bioelectric variation corresponds to loss of polarizability (depolarization); such an effect would accompany the breakdown of a polarizable partition. The return variation indicates return of polarizability (repolarization), implying reconstruction of the partition. The complete return of irritability during the relative refractory period usually occupies more time than the return phase of the electric variation (cf. Adrian, 1921); this apparently signifies that the newly reconstructed film does not immediately recover its former condition but requires to undergo some further change (see below).

Only a brief account of the bioelectric phenomena can be given here. A purely physical consideration of the possible conditions under which differences of electrical potential originate and undergo variation in living tissues would also be out of place in this textbook.[2] The essential underlying phenomena, in the physicochemical sense, are apparently phase-boundary potentials

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[1] For a general description of these phenomena, cf. Garten (1910); Bernstein (1912); Bayliss (1920), chap. xxii.

[2] Cf. Höber (1914), chap. xii.

(including membrane potentials); in the living cell these potentials are subject to variation in correspondence with the variability of the protoplasmic partitions. In systems containing electrolyte solutions and partitioned by thin, alterable semi-permeable films which are also the seat of chemical reactions (oxidations and reductions), electric phenomena of the kind observed are not surprising. The special features and the physiological rôle of the bioelectric currents depend on features of structure and chemical composition which are peculiar to organisms; but the general physical conditions underlying these phenomena are apparently of a kind well known in inorganic systems. This is indicated, for example, by the parallel between Macdonald's (1900) results with nerve and Beutner's (1920) with inorganic arrangements.

According to the present view, the bioelectric variations are the expression of variations in the electrical polarization of the protoplasmic membranes or surface films, especially the plasma membranes; these variations are the result of chemical and structural changes occurring in the membranes. This view is supported by the general nature of the conditions, both normal and artificial, under which the bioelectric currents originate. All artificial conditions which impair or destroy locally the semi-permeability of the plasma membrane lower the P.D. between the external surface of the cell and the interior and produce local bioelectric currents. These are known as "injury currents" and "demarcation currents." The conditions of observation and measurement of these currents in muscle and nerve are well known. In an uninjured resting muscle (e.g., frogs' sartorius) any two regions of the surface, connected through non-polarizable electrodes to a galvanometer, are typically found to be at the same potential (isoelectric); if then one region is injured (e.g., by cutting, heat, or chemical action) that region becomes negative relatively to the unaltered region, and a current flows through the galvanometer from the unaltered to the altered region. The observed P.D. varies with the extent and nature of the injury, but usually has a maximal value (at 20°) of 0.06–0.07 volt.[1] Since such an injury breaks down the plasma membrane locally and places the internal protoplasm in direct contact with the electrode (or with a solution in contact with the electrode), the most consistent interpretation of the electric effect seems to be that normally there exists a P.D. of this order (0.05–0.1 volt) between the outer and the inner surfaces of the plasma membrane; the injury, by doing away with the polarization at one electrode region, simply makes this P.D. evident. According to this conception the plasma membrane is normally the seat of a polarization, positive externally, the resting or physiological polarization. This polarization depends on the semi-permeability of the membrane; such a condition renders possible a permanent difference between the composition and concentration of the ions adjoining the two surfaces of the partition; to this difference corresponds a definite P.D. This conception regards the demarcation potential of cells as essentially a membrane potential, e.g., of the Donnan type. It seems probable, however, that superposed on the purely physical membrane potentials are potentials dependent on chemical conditions, especially oxidation-reduction potentials; this is indicated by certain general physiological facts such as the relation of oxygen to growth processes; more rapidly growing regions, where oxygen is more rapidly consumed, appear in general to be negative to slowly growing regions.[1] Typically, the concentration of oxygen is high outside and low inside the cell, and the constituents of the protoplasmic membrane are readily oxidizable. It is well known that the semi-permeability of the membranes depends on the continuance of vital processes which involve oxidations. Permanent loss of semi-permeability is a characteristic sign of death; correspondingly the demarcation potentials are absent or insignificant in dead tissues.[2]

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[1] For comparative data, cf. Garten (1910).

Since the local negativity induced by a cytolytic agent or mechanical injury is an expression of increased permeability, the fact that negativity is constantly associated with normal stimulation indicates that this latter process is also associated with an increase of permeability. This increase, however, must be temporary and reversible, in correspondence with the temporary and reversible character of the electric variation. Any temporary breakdown of the plasma membrane occurring at an excited region would be associated with a temporary negativity at that region and hence with a flow of current between excited and resting regions. This current would cease as the membrane was restored. A reversible negativity may in fact be readily produced by the brief local action of a permeability-increasing agent, e.g., by the application of a solution of KCl to a muscle; if the tissue is then washed with Ringer's solution the normal isoelectric condition returns, although more slowly than after stimulation. It is known that excessive stimulation may produce a more or less lasting local negativity (cf. Ebbecke, 1922) in a nerve or muscle; also that rapid cytolytic action has a stimulating or activating effect on many cells; so that the difference between the action of a stimulating and of a cytolytic agent is probably one of degree rather than kind. In both cases the return to the normal state is dependent on the reparative or constructive processes of the living protoplasm; these restore the temporarily altered membrane to its former state.

Theories of this general type are known to physiologists as the "membrane theories" of the bioelectric potentials; such theories regard the bioelectric variations of stimulation as the effect of temporary reversible changes in those structures, semi-permeable membranes, whose presence is a chief condition for the normal resting potentials (demarcation potentials, physiological polarization) of the irritable cell. The essential conditions in a muscle cell or nerve fiber locally altered by stimulation or otherwise may be compared with the conditions in certain simple types of inorganic system (or "models"), e.g., a galvanized (zinc-covered) iron wire immersed in acid and having some local interruption of its surface layer (R. S. Lillie, 1915). The film-covered and the altered regions of the surface are then at different potentials and a current ("local current") flow between the two. Such local currents may have further chemical effects; in some cases, as in passive iron immersed in nitric acid, they form the condition of a rapid transmission of chemical and electromotor effects to a distance from the immediately altered region. The passive iron model shows in many features of its behavior a close resemblance to irritable and transmissive protoplasmic systems like nerve (see p. 225). The cell surfaces, in the part which they play in the production of the bioelectric currents, exhibit properties which appear to be essentially the same as those of metallic electrodes. These resemblances will be discussed more fully below when the special conditions of transmission are considered.

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[1] For data and references, cf. R. S. Lillie (1923), pp. 399 ff.

[2] The precise physical conditions of membrane potentials may vary; cf. Beutner (1920), Loeb (1922), Bernstein (1912), Höber (1914). The essential point to be observed is that they cannot be maintained unless the membrane is impermeable to at least one of the ions present in either solution. Otherwise the chemical and electrical asymmetry necessary for the P.D. will soon disappear, as diffusion renders the conditions equal on the two sides of the membrane.

2. *Time relations of bioelectric variations:*

The electric variation resulting from a single stimulation of a particular species of muscle or nerve (e.g., with an induction shock) exhibits typically a range and a rate of development and subsidence which are characteristic for that tissue. The range of variation, i.e., the degree of potential change associated with stimulation, does not appear to differ widely in different tissues; this range is closely similar to that of the demarcation current (e.g., 0.04–0.07 volt). On the other hand, the time occupied by the variation in different tissues differs greatly. Typically, the rate of development (rise) is somewhat greater than that of subsidence; in general, the characteristic speed of reaction of the tissue, e.g., the rate of contraction in a muscle or the rate of transmission in a nerve, is closely related to the rate at which the bioelectric variation develops. This rate is also affected by temperature in the same manner as the physiological change; e.g., Lucas found that the temperature coefficient of the propagation velocity of the contraction wave in the frog's sartorius was almost identical with that of the rising phase of the bioelectric variation (Lucas, 1909). In general, when the bioelectric variation develops rapidly and is of brief duration, the response of the tissue to stimulation is also rapid; the muscle has a brief latent period and single twitch, together with a brief chronaxie, refractory period, and summation interval; the propagation of the excitation wave is also rapid. Conversely, slowly reacting and slowly conducting tissues have slow bioelectric variations. The rate of the electric variation represents under normal conditions a fundamental physiological constant of the tissue, which largely determines the rate of its other activities. The close correlation between the rate of rise of the electric variation in nerve (as measured by the string galvanometer) and the speed of the nerve impulse is indicated by the data in Table IV taken from observations of various investigators on the nerves of different animals (R. S. Lillie, 1914). The more precise significance of this relation between the speed of electric variation and the speed of propagation will be considered below.

TABLE IV

Nerve	Duration of Rising Phase of Action Current Curve in σ (.001 second)	Speed of Propagation of Excitation Wave
Frog's sciatic	0.9–1.2 (at 18°)*	*ca.* 30 m. per sec.
	0.55 (at 32°)	*ca.* 60 m. per sec. at 30°
Rabbit's sciatic	*ca.* 0.5 (at 32 °)	*ca.* 100 m. per sec. at 37°
Non-medullated (splenic) of horse	*ca.* 60–70	*ca.* 0.5 m. per sec.
Olfactory of pike	*ca.* 70 (at 12°)	118–150 mm. sec. at 13°
Mantle nerve of *Octopus vulgaris*	8.2–11.3	2.5 to 3.5 m per sec.
Commissural nerve of *Anodonta*	*ca.* 200	varying estimates; 1–5 cm. sec.

*Recent measurements with the cathode-ray oscillograph indicate an even more rapid rise (Gasser and Erlanger, 1922).

3. *Rhythm of bioelectric variations:*

Normal physiological rhythms, such as the rhythm of the heartbeat, are associated with bioelectric rhythms; and in rhythmical processes like tetanic contraction it can be shown that the bioelectric variation is also rhythmical and corresponds with the rhythm of excitation. These bioelectric rhythms are undoubtedly associated with chemical rhythms, but of the precise nature of the latter little is known at present. In cleaving sea-urchin eggs the rhythm of cell division is associated with a rhythm of CO_2 production (Lyon, 1904), apparently corresponding to the rhythm of membrane change, and probably a bioelectric rhythm is also present; evidence of an electric variation during cell division has in fact been obtained by Miss Hyde in the eggs of *Fundulus* (I. H. Hyde, 1904). It has been already mentioned that rapidly growing regions of plants and animals (i.e., regions of active cell division) are typically negative to slowly growing regions. These facts suggest that a rhythm of oxidations is associated with the bioelectric rhythms. A rhythm of heat production in the vertebrate heart has been recently demonstrated by Snyder (1922).

A tendency to rhythmicity is seen in many protoplasmic activities; this is illustrated, for example, by the wide distribution of ciliary movement at free cell surfaces in organisms of all kinds. Recently the natural bioelectric rhythms of mammalian voluntary muscle during contraction have been investigated in much detail. The usual rate is of the order of 50 per second; in the case of a given muscle it shows much constancy, although there appears to be considerable variation from muscle to muscle (Piper, 1912). The muscle rhythm corresponds to the rhythm of innervation, which in turn corresponds to the rhythm of discharge from the nerve cells of the central nervous system. Gasser and Newcomer (1921) have recently shown, by means of an amplifying arrangement, that the rhythm of the electric oscillation in the phrenic nerve during the normal respiratory movements corresponds exactly with the electric rhythm of the contracting diaphragm (70–100 per sec.); to each variation in the muscle corresponds one in the nerve, and presumably also in the nerve cells of the respiratory center. The remarkable uniformity of these oscillations indicates that the variations in the individual nerve fibers and nerve cells are synchronous and in phase with one another. Some unifying control, presumably electrical, evidently synchronizes the activity of the separate cells[1] of the center.

What is transmitted from a nerve fiber to a muscle cell in innervation is apparently simply a variation of electric potential, with the associated local bioelectric current. This view implies that the chief co-ordinating factors in the activity of the different elements in any neuromuscular mechanism (e.g., reflex arc) are the bioelectric variations. It can readily be shown by stimulating the motor nerve of any muscle by means of rhythmical induction shocks of known frequencies that the bioelectric variations of the muscle correspond with the electric stimuli applied to the nerve throughout a wide range of frequencies. An upper limit to this correspondence is, however, set by the duration of the refractory period of the muscle; if the frequency of the nerve stimulation becomes too high—i.e., above 200 per second with frog's muscle at 20°, and 1,000 per second with the muscles of warm-blooded animals—the muscular rhythm no longer follows the nervous rhythm but becomes irregular (cf. Höber, 1919).

Piper (1912) has shown that the normal bioelectric rhythm in the muscles of cold-blooded vertebrates has a temperature coefficient (at lower temperatures) of about 2. The following rates of oscillation per second were observed in the neck muscles of the tortoise, which were connected with a string galvanometer and caused to contract reflexly at the different temperatures.

Temperature	Oscillations per Second
4°	11
7°	15
12°	19
15.5°	25
18°	29
22°	35
26°	41
32°	51

Such a temperature coefficient indicates that the rhythm is determined by chemical reactions. The rate of development of single bioelectric variations shows a similar coefficient. The conclusion follows, if the electric variations of stimulation are the expression of changes in the membranes, that these chemical reactions are the ones which are concerned in the alteration of the plasma membranes.

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[1] The case of ciliated cells and of clumped spermatozoa beating in unison is apparently an instance of a similar control (cf. R. S. Lillie, 1914, pp. 428–29).

The phenomena of innervation apparently constitute a very clear illustration of the electrical control of normal cellular processes. The reason why the tetanic contractions produced by artificial rhythmical electrical stimulation and by normal innervation are physiologically indistinguishable is apparent; both are cases of rhythmical electric stimulation. In natural innervation the electrical rhythm originates in the motor nerve cells; the properties of these cells, fixed by heredity, determine their rhythm of discharge and hence the character of the resulting muscular contractions.

4. *Changes of membrane permeability or related structural changes during stimulation:*

The evidences of increase of permeability during stimulation which are afforded by the bioelectric variations and the refractory period are indirect; there are, however, many observations on other types of cell giving direct evidence of such increase. Normal or functional variations of permeability are frequent in both animals and plants; they are seen, for example, in gland cells during secretion, in egg cells after fertilization, in the pulvinus cells of sensitive plants during stimulation. In these processes, limiting membranes or partitions are apparently broken down more or less completely and then replaced. During cell division also the nuclear and plasma membranes undergo reversible breakdown or increase of permeability.

Instances where protoplasmic structure breaks down irreversibly (or undergoes progressive disintegration) as a result of some slight mechanical or chemical change are not unusual; this phenomenon is seen in the explosive blood corpuscles of crustacea, the platelets of vertebrates, nematocysts, and unicellular glands of various kinds; many other cells (germ cells, leukocytes, etc.) exhibit irreversible breakdown when cut or punctured by micro-dissection needles (see Sec. V). Such structural changes are apparently related to those underlying stimulation, with the difference that the reversal or recovery phase is absent. In true reversible stimulation, reconstruction follows automatically and rapidly upon the initial breakdown, so that there may be little external evidence of structural change.

Perhaps the clearest evidence of the dependence of stimulation on changes in the permeability of limiting semi-permeable membranes is seen in the turgor mechanisms of higher plants, especially the sensitive plant, *Mimosa*, and the Venus' fly-trap. In *Mimosa* the erect and expanded position of the petioles and leaves depends on the turgor of the pulvini; the cells of these structures are distended with water, with consequent stretching of the cellulose cell walls; the factors determining this distension are the osmotic pressure of the cell sap and the semi-permeability of the plasma membranes. When the plant is stimulated the cells immediately lose water and collapse, with resulting movements of the leaves. The water lost from the cells contains dissolved material, showing that the plasma membranes have become permeable to substances which formerly were confined within the cell—presumably those substances which maintain the turgor. Temporary loss of semi-permeability (shown in wilting) occurs in many plant cells as a result of mechanical stimulation; "stimulatory plasmolysis" is an example of the same effect. Recently B. Sen (1923) has shown that the electrical conductivity of both motile and non-motile plant tissues (pulvini, petioles of various plants) may be greatly increased (by 35–50 per cent) by stimulation. Electrical stimulation, summation, and anaesthesia occur in motile plant tissues under the same conditions as in irritable animal tissues; bioelectric variations are also characteristic, and there is a prolonged refractory period. The relation of change of permeability to stimulation seems particularly clear in plants, because the existence of turgor in the resting cells demonstrates unmistakably the impermeability of the plasma membranes to the crystalloid compounds contained within the cell; their sudden exit during stimulation can mean only increase of permeability.

In rapidly responding animal tissues like muscle and nerve the evidence of increase of permeability during stimulation is less direct; it is seen, however, in the decreased electrical polarizability observed at this time; this phenomenon has been known since Hermann's time (cf. Bernstein, 1912, chap. vii). In the more gradual forms of reaction, such as the response of the egg cell to fertilization or artificial activation, an initial increase of permeability can often be clearly demonstrated; in the sea-urchin egg there is increased electrical conductivity, readier entrance of dyes, increased loss of catalase; in other cases there is rapid loss or secretion of material from the surface layer or "cortical zone" of the egg (*Nereis*, lamprey); in the *Echinarachnius* egg definite changes in the plasma membrane (loss of coherence) can be shown to occur immediately after insemination (cf. Sec. VIII). Cell division in echinoderm eggs is accompanied by similar changes, both the semi-permeability and the coherence of the plasma membrane undergoing temporary decrease at the time of appearance of the cleavage furrow (R. S. Lillie, 1916).

In general, all of those physical or chemical agents whose primary effect is to increase permeability have a stimulating or activating effect on irritable cells or cell systems. In nerve or muscle any rapid local increase of permeability is equivalent to a stimulation. The activation of unfertilized eggs by cytolytic substances of all kinds is described in Section VIII; these substances also act as strong stimuli on many irritable systems, such as sensory nerve endings. This effect is well shown in frogs' voluntary muscle, especially after sensitization in pure solutions of Na salts; saponin, lipoid solvents, foreign blood sera, and soaps then cause vigorous contractions (R. S. Lillie, 1911). Pure (unbalanced) salt solutions, e.g., isotonic solutions of NaCl and other

Na salts, have a stimulating effect on many irritable forms of protoplasm, including muscle and nerve; they also activate echinoderm egg cells. It is well known that these solutions characteristically increase permeability, and that the permeability-increasing effect is prevented by the addition of a little Ca salt which also prevents the stimulating and activating effects. Thus Ringer (1886) observed that, in pure isotonic NaCl solution, frogs' muscles twitch spontaneously, but cease when a trace of $CaCl_2$ is added to the solution. The larva of the marine annelid *Arenicola* is a favorable organism for demonstrating effects of this kind; this larva (a segmented trochophore $\frac{1}{3}$ mm. long) contracts strongly and persistently in an isotonic solution of NaCl (or similar Na salt) and gives evidence of a general increase of permeability by loss of pigment, but in NaCl solution containing $CaCl_2$ ($20 NaCl + 1 CaCl_2$) both effects are slight or absent (R. S. Lillie, 1909); various anaesthetics also prevent simultaneously the stimulating and the permeability-increasing effects of the pure salt solution (R. S. Lillie, 1913). Similarly, pure solutions of various Na and K salts cause membrane formation and partial activation in starfish and sea-urchin eggs, accompanied by evidence of increase of permeability (loss of pigment); both the activating and the permeability-increasing effects of the solutions are prevented by adding $CaCl_2$. Pure isotonic solutions of $MgCl_2$ and $CaCl_2$, whose initial effect is to decrease permeability, are without activating effect (R. S. Lillie, 1910, 1911).

The general nature of the structural change associated with stimulation in contractile forms of protoplasm appears to be illustrated in the effects produced by pure isotonic salt solutions on the ctenophore swimming plate (R. S. Lillie, 1906); this is a large compound cilium, exhibiting in a simple form many of the essential properties of irritable and contractile protoplasm. When a detached row of plates is placed in a pure or slightly acidulated isotonic salt solution (of NaCl or similar salt), the contractile rhythm is greatly accelerated and the vigor of the beat increased, the plate behaving as if intensely stimulated. This period of intensified activity lasts for only a short time (about one minute), and is associated with a progressive whitening or coagulation of the originally clear protoplasm and a loss of the coherence of the separate cilia, many of which vibrate independently; when these changes have progressed to a certain stage the plate ceases movement; examination then shows it to be dead and structurally disorganized, so that it falls to pieces on shaking. Without this period of intense activity no such rapid structural change occurs. The indications are that during contraction some interfibrillar material or film structure, which is necessary to the normal coherence and co-ordinated activity of the plate, is broken down or loses structural continuity. Apparently under the abnormal conditions just described the reconstruction or recovery stage of the stimulation cycle is incomplete; accordingly there is a progressive loss of structural coherence with consequent disintegration. Coagulation indicates the coalescence of protein particles to form larger aggregates; the increase of permeability is shown in the later changes undergone by the altered structure (swelling in sea water).

The phenomena of light production in luminous animals (such as the firefly) also afford indirect evidence of the breakdown of film structure during stimulation. Dubois and Harvey have shown that the photogenic effect depends on the union of the two compounds, luciferin and luciferase, in the presence of oxygen (Harvey, 1920). These compounds are present in the single photogenic cell; on stimulation they unite and yield the luminescent reaction. Evidently they are kept apart in the living resting protoplasm, presumably by the interposition of partitions or films; when these films break down in stimulation, the compounds are free to interact. The production of light in the stimulated photogenic cell, as well as the bioelectric variation, is thus the expression of a temporary breakdown (or increase of permeability) of film structure.

5. *Refractory period:*

The chief phenomena of the refractory period in irritable tissues have already been described briefly; these phenomena also afford evidence of changes in the polarizable partitions (membranes) of the irritable system. On the present theory we should expect variations of sensitivity to run parallel with the structural changes accompanying stimulation. The evidence afforded by the time relations indicates that the upstroke (rising phase) of the bioelectric variation corresponds to the period of complete or almost complete inexcitability (absolute refractory period); during this period the polarizable membranes concerned in stimulation apparently undergo temporary alteration or breakdown involving loss of semi-permeability. This change involves loss of polarizability and hence of irritability. Apparently, irritability begins to return (at least in muscle and nerve) during the downstroke (return phase) of the electric variation; the reversal of the electric variation probably signifies reconstruction of the membrane. On this view we can understand why the refractory period is prolonged by conditions, such as fatigue, lack of oxygen, cyanide, various poisons, that interfere with processes of repair. The refractory period has frequently been compared to a brief period of fatigue (Verworn, 1913). The fact that the relative period usually lasts several times longer than the return phase of the bioelectric variation (repolarization phase) has already been mentioned. It is interesting to note that in this respect the refractory period of living tissues resembles the period of imperfect transmissivity immediately following automatic repassivation in the passive iron model (see below). In this case the newly deposited film of oxide is at first relatively resistant to alteration by local electric currents, and requires some further time before it is in a state permitting of the ready transmission of activation to a distance. If the conditions in the living tissue are comparable with those in the metallic model, we may infer that the relative refractory period corresponds to the time during which the newly re-formed protoplasmic film is undergoing some further change of structure or composition which increases its alterability by the electric current.

6. *Chemical changes and heat-production in stimulation:*

The chemical changes resulting from stimulation cannot be considered in any detail in this section. Each irritable cell or tissue has its own specific activities and metabolism, both of which are altered in a characteristic manner in stimulation. Usually the consumption of oxygen and evolution of CO_2 are increased; yet in nerve, the simplest irritable tissue, this effect is so slight that its demonstration is difficult and uncertain. In the absence of oxygen, however, vertebrate nerve is fatigued by repeated stimulation, and recovers only when oxygen is restored; and Waller (1896) and Tashiro (1917) have found evidence of increased output of CO_2 during stimulation. The almost entire absence of heat production in this tissue seems to indicate that the chemical changes associated with stimulation are very completely reversed, and that there is little permanent alteration of material during the stimulation cycle. The need of oxygen for recovery indicates that oxidations are chiefly concerned in the reversal or reconstruction phase of the cycle; this is very clearly true of other irritable tissues, especially muscle (cf. A. V. Hill, 1922).

The relations of the chemical reactions and heat production of the stimulated cell to the characteristic response have been most completely investigated in the case of vertebrate voluntary muscle; and much light has been thrown on the general features of the chemical cycle underlying contraction and relaxation by the recent work of Hill and Meyerhof.[1] In the muscle cell the surface energy of the colloidal elements forming the fibrillae is apparently transformed into the mechanical energy of contraction as a result of stimulation, the essential physical change being a rise of surface tension under the influence of lactic acid formed from the carbohydrate of the muscle cell. The segmented structure of the fibrillae in striated muscle allows the movement to occur rapidly with a minimum of interference from friction or the inertia of displaced fluid (R. S. Lillie, 1912). The most evident chemical changes occurring in the muscle cell, the increase in lactic acid during stimulation and its disappearance as a result of later oxidation processes, have already been briefly described. The production of lactic acid and the contraction both occur readily in lack of oxygen, i.e., are anaërobic processes; but the reversal phase of the cycle, in which lactic acid is removed and irritability and contractility are restored, is intimately dependent on the presence of oxygen. The time relations and other features of the production and disappearance of lactic acid in the contraction cycle, and also of the accompanying phenomena of heat production, are described in Hill's recent reviews (1914, 1922) on the mechanism of muscular contraction. The primary phase of the cycle, that of stimulation and contraction, is independent of immediate oxidations. The production of acid is apparently definitely localized with the cell, most probably at the interface between the contractile fibrils and the sarcoplasm. The probable sequence of events in the single contractile cycle may be pictured somewhat as follows. The excitation wave, originating at the cell surface (motor end plate), travels over the surface of the fibrils; like other excitation waves it is associated with a chemical change in the interfacial films there present; this change involves the temporary breakdown of these films and the formation of lactic acid; under the influence of the acid the surface tension of the fibrils (or of their finer colloidal elements) is increased and contraction results. The recovery or reconstruction phase then immediately follows, during which the film is restored; in this process the lactic acid is neutralized and removed, partly by oxidation to CO_2 and water; this oxidation furnishes the energy for the structural and chemical changes of reconstruction, including a reversion of the greater part of the lactic acid to the carbohydrate stage. The system is then in a position to repeat the cycle. The energy of contraction is thus derived ultimately from the energy of oxidation of carbohydrate (the precursor of lactic acid). For the probable details of the chemical cycle the student should consult the papers of Hill and Meyerhof. The chief production of heat in the contractile cycle follows the contraction, and is associated with the oxidative processes underlying recovery. Heat is produced during the onset and maintenance of the contraction and also for several minutes subsequently. The evidence indicates that only a small proportion (about one-fifth or one-sixth of the lactic acid freed during the excitation process) is oxidized completely in the recovery process, the remainder being regenerated to carbohydrate (probably glycogen). In a recent paper by Hill and Lupton on muscular exercise, the chemical cycle of the muscle cell is compared with that of an accumulator (Hill and Lupton, 1923, p. 139).

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[1] For references, cf. Hill (1922).

> The muscle is to be regarded as an accumulator of energy, energy available for rapid non-oxidative discharge, stored during previous oxidations. The transformation of glycogen into lactic acid, the action of the lactic acid on the muscle proteins, and the neutralization of the lactic acid by the alkaline buffers of the muscle, are the vehicle by which this stored energy is made manifest; during recovery the process is reversed at the expense of a portion of lactic acid oxidized. The accumulator has been recharged at the expense of oxidations required to run the dynamo. We must regard the muscle, therefore, as possessing two mechanisms: (*a*) the anaërobic one of discharge and (*b*) the oxidative one of recovery. . . . .[1]

We have considered the special case of muscle in some detail, because what is true of the muscle cell is probably true, in a general sense, of all irritable cells having reversible stimulation cycles.[2] In nerve also there is evidence that the process of breakdown underlying the transmitted disturbance differs from the process of recovery in a manner very similar to that just outlined for the case of muscle. Transmission and recovery appear to depend on chemically different processes; there is evidence of a higher temperature coefficient (Q_{10} approaching 3) for the recovery process (Adrian, 1921), and its dependence on oxygen is greater. Little, however, is known regarding the details of these processes; their initiation and control by the electric current and their susceptibility to surface-active substances (narcotics) are a general indication that the essential reactions occur at the structural surfaces of the irritable protoplasm.

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[1] See also Hill's recent Royal Institution Lecture (Hill, 1923).

[2] An instructive case is that of the light receptor elements described in Hecht's recent studies of the stimulation and recovery process in the light reactions of mollusca (Hecht, 1920, 1921).

VII. CONDITIONS MODIFYING REACTIVITY OF CELL

In all irritable systems reactivity is subject to modification under various normal and artificial changes of condition. The variations of irritability during the refractory period, fatigue, and sleep are examples of normal variations. There are also variations connected with the nutritional state of the cell; e.g., well-fed animals no longer respond to the presence of food, while starved animals become abnormally responsive. Many external conditions, including temperature, chemical and osmotic conditions in the surrounding media, electrical conditions (the passage of constant currents through the system), also affect the irritability and rate of reaction of cells. Reversible influences of this kind—those which modify activity or irritability without injury to the system—are of chief physiological interest; and a brief review of the effects of the foregoing agencies on irritability and automatic activity will now be given.

1. *Temperature:*

Temperature affects reactivity chiefly by its direct influence on the rate of the underlying chemical and physical processes. It may also act by altering the structural or other conditions in the irritable system, e.g., by changing the physical state of the colloids. In some cases changes of temperature themselves act as stimuli or activating agents; contact of "hot" or "cold" bodies—i.e., bodies at temperatures outside the normal physiological range—stimulates many forms of protoplasm. In the temperature sense organs of warm-blooded animals, the sensitivity to slight changes of temperature has been developed to a high degree. The unfertilized eggs of the starfish may be activated by temporary exposure to either high (30°–38°) or low temperatures (below 6°); similar facts are known for other eggs, e.g., *Nereis* (cf. Sec. VIII).

The influence of temperature on the various processes included in the stimulation cycle of a muscle or nerve—latent period, muscular twitch, bioelectric variation, transmission of excitation, refractory period—is in general uniform; within the physiological range the time occupied by each process is shortened as the temperature rises, usually in a ratio between 2:1 and 3:1 for a rise of

10° ($Q_{10}=2-3$). This ratio of velocity increase for a given rise of temperature (or temperature coefficient) is characteristic of chemical reactions; the result indicates a dependence of the physiological processes on the chemical reactions of protoplasm.[1]

The precise values of the temperature coefficients of the several processes which succeed one another in the stimulation cycle appear to be somewhat different; these differences are of interest since they afford indication of the physical nature of the processes and hence aid in the physiological analysis of the cycle. Lucas (1910) found the summation interval for inadequate stimuli to be lengthened in the ratio of only 1:1.3 by a change from 18° to 8°. This low Q_{10} value is similar to that of diffusion, and indicates that the primary effect of the current is purely physical, consisting probably in the transport of ions to the semi-permeable surfaces. A distinctly higher Q_{10} value (1.7–2.0) is found for the velocity of transmission of the excitation wave or nerve impulse (cf. Kanitz, 1915, p. 67); this value is significantly lower than that of most other physiological processes and of the generality of chemical reactions. A possible explanation of this low value is that transmission depends on a combination of an initiatory chemical reaction with a succeeding purely physical process of structural breakdown, the latter having a temperature coefficient similar to that of diffusion. A process consisting of a brief chemical change followed by a purely physical disintegration would have a Q_{10} value intermediate between those of diffusion and of chemical reactions. The highest Q_{10} value (2.5–3) is found for the recovery process, which occupies the refractory period (Bazett, 1908; Adrian, 1914, 1921); this result indicates the predominantly chemical nature of the processes underlying recovery.

The influence of temperature on sensitivity as such, i.e., on the susceptibility to stimulation or activation by a definite stimulus of constant value, should also be noted. Many irritable tissues show marked decline of sensitivity when cooled below a certain temperature; this effect is familiar in the muscles and nerves of warm-blooded animals (anaesthesia by cold); it is also seen in the tissues of tropical marine animals (Mayor, 1914). Many tissues also lose sensitivity toward the upper limit of the physiological temperature range; the "heat standstill" of the frog's heart is an illustration. Similarly, the musculature of tropical medusae becomes irresponsive at 40° and recovers on lowering the temperature (Harvey, 1910). Frogs' muscle and nerve when cooled show increased excitability to electric currents of long duration; i.e., the threshold intensity is lowered (Lucas and Mines, 1907). This effect is probably complex, but the polarizing effect of a current of given intensity will be greater at the lower temperature because of the slowing of the counter-diffusion. In the activation of the unfertilized starfish egg by butyric acid a marked facilitation of the process is seen at temperatures approaching 25°;

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[1] For a general review of the temperature-coefficients of physiological processes, cf. Kanitz (1915).

rise of temperature shortens the necessary exposure to a much greater degree than would be expected from the chemical temperature coefficient alone (R. S. Lillie, 1917). This effect probably depends chiefly on a structural alteration which facilitates the penetration of the acid; incipient heat activation (depending on the increased production of acid by the protoplasm itself) probably also plays a part.

2. *Electrical conditions:*

The polar effect of the electric current on irritable tissues (muscle and nerve) is seen not only in stimulation at the polar regions at times of sudden changes of intensity, but also in characteristic changes of irritability during the flow of the current. These effects have long been known under the name of electrotonus; constant currents of moderate intensity induce heightened irritability in muscle and nerve in the neighborhood of the cathode and the reverse condition (depressed irritability) near the anode. In a spontaneously active tissue such as the heart the influence at the anodal region is inhibitory, contraction being prevented wherever the current density is sufficient. In conducting tissues like nerve the transmission of the excitation wave is blocked. Electrotaxis in Protozoa and galvanotropism in growing plants and animals are closely related phenomena which also depend on the polar action of the current; e.g., in *Paramoecia* the ciliary stroke is reversed on the surface facing the cathode. The directive influence of the current on growth (galvanotropism in plants and hydroids) has probably a similar basis.

All such effects, as well as the stimulating effects already described, are to be referred to the influence of the current on the electrically polarized semipermeable surfaces of the irritable elements. At the region where the current (positive stream) enters the living system, i.e., near the anode, the physical effect on the plasma membranes is an increase or reinforcement of the preexisting or physiological polarization; near the cathode the effect is of the reverse kind (i.e., decrease of polarization). To this contrast in the physical effect corresponds a contrast in the physiological effect. Apparently in the typical irritable element, such as a nerve fiber, any increase of external positivity renders stimulation difficult, and vice versa. The change thus produced at the anodal region is contrary in direction to that associated with or determining stimulation, hence interferes with the latter. A similar influence of the constant current is seen in the passive iron model (R. S. Lillie, 1920, p. 136); when the passive wire (immersed in dilute nitric acid) is rendered anodal, it becomes more resistant to mechanical or other activation. In this case the effect depends on an increase of the oxidative action which normally preserves the passivating film intact; the addition of an electrochemical oxidizing influence to that of the nitric acid increases the rate of oxidation and has the effect of stabilizing the film. Conversely, when a passive wire is rendered cathodal (with a current too weak to activate) mechanical activation is more readily induced than when no current is flowing. Analogy would suggest that in the irritable living system (nerve fiber or muscle cell) any increase of positive polarization favors or promotes those reactions (presumably oxidative) which maintain the continuity and stability of the surface film; hence processes like stimulation, which depend on breakdown of the film, are opposed or prevented. In general, any condition which stabilizes the protoplasmic surface film decreases irritability; this effect is seen especially in the phenomena of narcosis (see below). Conversely, sensitization corresponds to a decreased stability of the surface film.

3. *Chemical and osmotic conditions in the medium:*

Typically, a necessary condition for normal cellular reactivity is a certain definite composition and concentration of the dissolved materials in the external medium. In the case of the cells of higher animals especially, an approximate constancy of osmotic pressure, salt content, and H-ion concentration is necessary to normal irritability and spontaneous activity. Deviations from this norm occasion corresponding variations in these properties. The phenomena vary greatly in detail, and only the more general features of these effects can be considered.

4. *Salt content:*

The media of all living cells contain neutral salts in solution, chiefly chlorides of Na, K, and Ca; and variations in the salt content have corresponding effects on irritability. In the case of most marine animals the concentrations and proportions of the salts resemble closely those of sea water. In teleost fishes and in air-breathing vertebrates, while the proportions of the chief salts remain very nearly the same as in sea water, the concentrations are much lower, and an approximate constancy of osmotic pressure is maintained by the regulatory activity of the kidneys.

In general there are three chief conditions, affecting the activity and irritability of the cell, which are directly dependent on the neutral salts of the medium: (1) the properties of the plasma membrane, e.g., its physical state or consistency and its permeability, (2) the electrical polarization of the cell surface, and (3) the electrical conductivity of the medium. Variations in all of these conditions influence irritability and the rate or character of the response to stimulation.

With regard to the first condition, it is now known that semi-permeability depends on the presence of the external salts in balanced proportions; removal of calcium especially soon leads to increased permeability and eventual death; the toxicity of pure NaCl solutions is thus explained, as well as its antagonism by salts of Ca and similar metals. Structural changes in the plasma membrane are at the basis of these effects; ultimately such changes are to be referred to the influence of the salts on the physical state of the structure-forming colloids (proteins and lipoids) of the membrane.[1]

In the chief irritable elements of higher animals (muscle, nerve) each of the three chief cations (Na, Ca, K) appears to have a specific influence in the preservation of the normal properties. Irritability is directly dependent on the presence of Na salts in somewhat high concentration. When the tissue is placed in a medium containing the other salts in normal proportions, but sugar (or other indifferent non-electrolyte) in place of NaCl, irritability soon disappears; if the tissue is then placed in a pure solution of a Na salt, irritability promptly returns (Overton, 1902). Lithium is apparently the only cation which can replace sodium in this relation. Even in the presence of sufficient Na salt, normal irritability and working power are not long maintained in the absence of either K or Ca; this is well shown in the case of the frog's heart, which soon weakens when perfused with media deficient in either salt (Clark, 1913; Clark and Daly, 1921). It is remarkable that potassium, whose presence in low concentrations is necessary to the maintenance of irritability, has a strongly paralytic action in higher concentrations. Rb and Cs can partly replace K in its action as maintainer of normal irritability in presence of Na and Ca salts (Clark, 1922).

5. *Sensitization and desensitization by salts:*

When a frog's muscle is placed for a few minutes in a pure solution of NaCl (or similar Na salt), its irritability to chemical agents (K salts, lipoid solvents, etc.) is found to be greatly increased; i.e., the tissue is sensitized (R. S. Lillie, 1911). This effect is readily reversed in Ringer's solution. It is not produced by Na-salt solutions to which a little Ca has been added. Isotonic $CaCl_2$ and $MgCl_2$, on the contrary, cause reversible depression of irritability or desensitization, an effect analogous to anaesthesia. A remarkable form of "contact sensitivity" is produced in both muscle and nerve by immersion in solutions of Na salts whose anions precipitate Ca, or decrease the concentration of Ca ions, such as tartrate, citrate, sulphate, oxalate; the tissue then becomes hypersensitive to contact of air, mechanical stimulation, or similar influences (J. Loeb, 1901). Normal irritability returns in Ringer's solution. Since the action of the salts is evidently superficial, such effects indicate that any modification of the physical or chemical state of the plasma membrane has a corresponding modifying effect on irritability. The special physiological or pharmacological effects of different salts and other chemical agents are undoubtedly to be referred largely to similar influences exerted upon the plasma membranes.[2]

6. *Importance of electrolytes in medium:*

It has long been known that irritable tissues such as muscle and nerve lose irritability in isotonic solutions of sugar and other indifferent non-electrolytes;

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[1] For a review of the physiological action of salts, cf. Höber (1914), chaps. x, xi.

[2] For a fuller discussion, cf. R. S. Lillie (1923), chaps. viii, ix.

this effect is not toxic, but is a simple consequence of the withdrawal of salts; irritability promptly returns in Ringer's solution or solutions of Na salts (Overton, 1902). Such experiments again illustrate the dependence of irritability on the salt-content of the medium. The effect is complex; in addition to the specific "sodium effect" just described, the change of surface polarization in the muscle cells and the decrease of electrical conductivity are probable factors. An especially significant fact is that in mixtures of isotonic sugar solution and Ringer's solution the rate of propagation of the contraction wave is decreased, in close proportionality to the electrical conductivity of the medium (Pond, 1921). At concentrations of *ca.* 0.05 per cent NaCl the tissue ceases to respond to stimulation. Mayor also observed that in dilute sea water the rate of transmission in the nerve net of the medusa Cassiopeia was directly proportional to the conductivity of the medium (Mayor, 1917). This general result is consistent with the theory that normal transmission is dependent on the passage of electric currents (the bioelectric currents) through the extracellular media. It is to be presumed that if the conductivity of the medium falls below a certain minimum, excitation is no longer transmitted; the inexcitability of the tissue in such media is thus explained.

7. *Osmotic pressure of medium:*

Changes of external osmotic pressure also influence irritability, presumably through their effect in changing the water content of the cell. A rapid change of this kind acts as a stimulus; thus frog's motor nerve is stimulated by dipping in solutions (of indifferent substances) of about double the normal osmotic pressure (Mathews, 1904). The parthenogenetic action of hypertonic sea water on sea-urchin eggs is apparently a phenomenon of a related kind, but its physiological basis is insufficiently understood.[1] A promoting influence on certain chemical reactions in the egg protoplasm, presumably syntheses, appears to underlie the effect; this is indicated by its dependence on oxygen and its high temperature coefficient (J. Loeb, 1913). The influence of variations of osmotic pressure on the automatic activity of cells is also a fact of general interest, well illustrated in the case of the vertebrate kidney; slight decrease in the normal osmotic pressure of the blood promotes secretory action while slight increase inhibits it. In irritable tissues in general (muscle and nerve) moderate dilution frequently increases irritability while increase of concentration diminishes it; this is also the case for the spontaneous activity of the *Limulus* heart ganglion and strips of vertebrate heart muscle (Carlson, 1906). The conditions in nerve are different; transmission is most rapid at the normal osmotic pressure of the medium and is decreased by a change of concentration in either direction (Shoji, 1919). The response of smooth muscle appears to be especially susceptible to changes of osmotic pressure; thus Dale found that the action of adrenalin and β-iminoazolyl ethylamine is greatly modified by even slight changes of osmotic pressure.[2]

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[1] Cf. R. S. Lillie (1915), p. 300; (1922), p. 272.

[2] Cf. Bayliss (1920), pp. 162 ff.

8. *Hydrogen-ion concentration of medium:*

In most cells the maintenance of a normal H-ion concentration in the medium is also an essential condition for normal irritability. Certain forms of irritability and spontaneous activity are highly sensitive to variations of H-ion concentration; among animals this form of reactivity has attained its highest development in the respiratory center of vertebrates, the cells of which increase their rhythm of discharge under an increase of H-ion concentration too slight to be detected by any known chemical indicator; upon this special responsiveness the regulation of the breathing movements depends.[1] On the other hand, the excitability of nerve trunks seems to be only slightly modified by variations of H-ion concentration within a considerable range ($pH = 9–4$); electrical sensitivity decreases between pH 7 and pH 4 as acidity increases, but the effect is gradual. The course of recovery after stimulation is, however, modified in a remarkable manner in media of this degree of acidity, the nerve becoming hyperexcitable for a brief period (some hundredths of a second) after stimulation (Adrian, 1920). In skeletal muscle stimulated through nerve, responsiveness is not noticeably affected by variations of H-ion concentration between pH 5 and pH 8; but outside this range, on either the acid or alkaline side, the extent of the muscular contraction is rapidly decreased and the tissue is more readily fatigued by stimulation. On the alkaline side between pH 10 and pH 9 the muscle becomes temporarily more excitable than normal (Grant, 1920). In automatically rhythmical tissues, increase in the rate of rhythm appears to be a frequent effect of slight increase of H ions; this was observed by Bethe (1909) in the beat of medusae and by Carlson (1906 *b*) for the *Limulus* heart; the well-known rhythmical twitching of vertebrate skeletal muscle in pure NaCl solutions is also favored by slight acidulation (J. Loeb, 1899). In the frog's heart, however, even a slight increase in the acidity of the perfusing solution soon diminishes both the strength and the rhythm of the beat (A. J. Clark, 1913).

In many cases the effects of slight addition of strong acid to the media may be referable to the resultant increase in the tension of CO_2; such increase has been shown to have a sensitizing effect on certain tissues, e.g., nerve (Waller, 1896). The activation of starfish eggs by adding mineral acids to the sea water may be in part an effect of increased CO_2 tension; the parthenogenetic effect of CO_2 and fatty acids is largely independent of the external H-ion concentration. An interesting and not infrequent effect is the reversal of heliotropism by acid; this occurs in *Arenicola* larvae at pH 6–5 in carbonate-free media, and apparently depends on the conversion of the stimulating action of light into an inhibitory action (Lillie and Shepard, 1923).[2]

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[1] The specific action of CO_2 or of the carbonate ion is also probably a factor in this effect; cf. Wilson (1923).

[2] The literature of H-ion action is very large; cf. Clark (1922), Wilson (1923); also the textbooks of Bayliss and Höber for further facts and discussion. It should be noted that the physiological H-ion effects are closely related to physicochemical effects, e.g., those having relation to the isoelectric points of the proteins and other biochemical compounds. The physical and chemical properties of proteins vary with H-ion concentration (cf. J. Loeb, 1922).

9. *Action of surface-active compounds. Narcosis:*

The reversible decrease or abolition of irritability and automatic activity (rhythmical action, cell division, growth) under the influence of the surface-active or lipoid-solvent group of compounds (the organic anaesthetics) is a universal phenomenon in living matter. It is important to note that this physiological effect is not peculiar to organic compounds but may also be produced by inorganic salts (e.g., of Mg, Ca, K), or by removal of electrolytes from the medium, or by certain physical changes of condition (cold, electrical polarization). The case of organic anaesthetics is, however, of particular interest, since it throws definite light on the conditions controlling the chemical reactions immediately concerned in stimulation.

The narcotic effectiveness of different organic compounds varies widely, and appears to be correlated with their physical rather than with their special chemical properties. This is indicated by the fact that compounds of the most diverse chemical classes (alcohols, ethers, esters, amides, nitriles, ketones, normal and substituted hydrocarbons, ethylene and other unsaturated compounds, etc.) have narcotizing effects; and also by the fact that the effectiveness of a compound has no very definite relation to the special nature of its chemically characterizing group; i.e., the narcotizing power of a particular alcohol, ester, ketone, or other chain compound depends chiefly on the length and other characteristics of its carbon chain, and only slightly on the nature of its polar group. On the other hand, the solubilities and the degree of surface activity have a close connection with the narcotizing properties (Overton, Traube, Czapek, Warburg).[1]

The most significant general fact is that in any homologous series the narcotizing effectiveness of a compound (as measured by the reciprocal of the narcotizing concentration) increases regularly with the increase in molecular weight, and shows a general parallelism both with the solubility of the compound in organic solvents (as indicated, e.g., by the oil-water partition ratio) and with its surface activity. The latter property is, in general, a measure of its tendency to undergo increase of concentration (adsorption) at the boundary surfaces between aqueous and non-aqueous phases. The question whether lipoid solubility or surface activity is the essential property on which narcotic action depends has been much debated. The case, however, is probably not one of alternatives; in general, the solubility in oil, lipoid, or other organic solvent increases as the solubility in water decreases; evidently the tendency to pass out of aqueous solution or to undergo adsorption at the surface of the non-aqueous phase depends on the same conditions as those determining solubility in water-immiscible organic solvents. In chain compounds with a terminal or intercalated active group, the proportion (in mass or volume) of the polar or water-combining group to the remainder of the molecule is apparently the chief factor determining the degree of adsorption as well as of organic solubility, hence both properties increase with the length of the carbon chain. In a system having the structure of protoplasm, in which the lipoids are apparently present chiefly as thin surface films, the distinction between adsorption and lipoid solubility ceases to be of importance.

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[1] For references, cf. my recent review, "The theory of anaesthesia" (R. S. Lillie, 1916); also Winterstein (1920); Höber (1914), chap. ix.

The Overton-Meyer theory refers narcosis to the solution of the narcotizing compound in the lipoids of the cell, while Traube, Czapek, and Warburg emphasize the importance of adsorption at the protoplasmic phase boundaries. The essential problem, however, is why such changes in the composition of the irritable system should render the latter temporarily insensitive to stimulation and decrease its automatic chemical and other activity.

Since the evidence already reviewed shows that stimulation is associated with a structural change in the irritable system—apparently in the nature of a temporary increase in the permeability of the protoplasmic surface films—it seems clear that the presence of narcotic compounds must in some manner prevent or interfere with this change. In other words, in the presence of these compounds, the films are rendered less susceptible to change than formerly, i.e., are *stabilized.* Stabilization of protoplasmic film structure would involve decreased susceptibility to stimulation. The structural change resulting from stimulation is, however, the expression or result of chemical change occurring under electrical influence. Any condition, therefore, which prevents this chemical change in the membranes will prevent the structural change and with it the associated stimulation.

It is in fact well established that increase of permeability, occurring under the influence of pure NaCl or similar solutions, may be checked or prevented when surface-active compounds in anaesthetizing concentrations are added to the salt solution. The stimulating or activating effect of the pure solution is prevented at the same time. This is well shown in *Arenicola* larvae (R. S. Lillie, 1913) and also in *Arbacia* eggs (R. S. Lillie, 1914). The anaesthesia of motile plant tissues, e.g., of *Mimosa* and *Dionaea*, in which the normal movement depends directly on increase of permeability, is a further indication that anaesthetics act by preventing structural changes on the membranes. Other instances of stabilization of protoplasmic film structure by surface-active compounds are well known.[1]

In those non-living heterogeneous systems where oxidations are catalyzed at phase boundaries, as in suspensions of charcoal containing amino acids or other oxidizable compounds, it is known that the presence of surface-active substances reduces the velocity of these reactions to a degree which is closely proportional to the quantity of the surface-active compound adsorbed. Warburg's recent investigations on the charcoal system illustrate this effect with great clearness; they show, for example, that the anti-catalytic effectiveness of the different members of the same homologous series is closely proportional to their surface activity; the same is true for the influence of the same compounds on oxygen consumption by living cells. Apparently the anti-catalytic effect is the result of displacement of the oxidizable compound (cystin, oxalic acid) from the surface of the charcoal by the more readily adsorbed narcotic compound; that this occurs could be shown quantitatively in certain cases; for example, when a certain proportion, e.g., one-half, of the adsorbed cystin was displaced from adsorption by the addition of a certain quantity of urethane the rate of oxygen consumption was reduced in almost the same proportion (Warburg, 1921, 1922).

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[1] For a fuller discussion and references, cf. R. S. Lillie (1923), chap. ix.

We may assume that in the living system surface-active narcotic compounds have a similar action, displacing dissolved substances of a lower degree of surface activity from the protoplasmic surfaces. If these displaced substances are the ones upon whose reaction the structural change of stimulation depends, stimulation will thus be interfered with or prevented. We may suppose, for example, that the increase of permeability accompanying excitation depends directly upon the transformation of adsorbed carbohydrate to lactic acid; then the displacement of the carbohydrate by some indifferent surface-active compound, such as ether or urethane, will make the critical membrane change temporarily impossible. The system is then rendered incapable of responding to stimulation or of transmitting states of excitation. When the compound is removed, the system regains its former properties.

Some such conception of narcotic action seems indicated by recent research, and is consistent with a wide range of biological and physicochemical fact. While the details of the chemical reactions determining the stimulation of special irritable systems are imperfectly known, there can be no doubt of their dependence on protoplasmic structure. We have already reviewed the evidence indicating that structure affects chemical reactivity mainly through its surface influence. The electric current, by its polarizing action at the protoplasmic structural surfaces or phase boundaries, alters the conditions of chemical reaction in those regions; hence its stimulating, inhibitory, or other effect. The facts of stimulation show that the action of the living system is intimately dependent on the distribution of the oxidizable and other reactive compounds of protoplasm in relation to the surfaces of the protoplasmic structures. Whatever alters this distribution alters reactivity; hence compounds with a special tendency to attach themselves to surfaces have a special action of this kind. On this property their effectiveness as anaesthetizing agents depends.

It has already been pointed out that other conditions (temperature, presence of certain salts, cyanide, electrical influence) may have depressant or narcosis-like effects; these effects are also to be considered in any complete discussion of the problem of anaesthesia. Stabilization of protoplasmic structure appears to be the general or fundamental condition. This stabilization can be produced by a direct interference with metabolism, as in the case of cyanide, as well as by other means. For a more detailed account of the facts and theories of narcosis and for the literature the more special articles and treatises should be consulted.

VIII. CONDUCTION OF EXCITATION IN PROTOPLASM

It has been pointed out that the total effect of stimulation depends upon transmission of excitatory influence from the region directly affected by the stimulus to the other regions of the irritable system. Brief reference has also been made to evidence indicating that the main conditions of this transmission are electrical; i.e., that in a transmissive element such as a nerve fiber the electric change at any active region is the factor which determines the transmission of the state of activity to the adjoining region. The association of a high degree of electrical sensitivity with a rapid rate of transmission in such a tissue as nerve is itself an indication of a close interconnection between the two properties. An active area of a tissue is always the source of an electric current which traverses the adjacent resting area of the tissue, as well as the surrounding medium; and the hypothesis that this current excites electrically the latter regions, and thus serves to transmit the state of excitation, is one which naturally suggests itself. The rapidity of transmission in nerve and muscle seems too great to be accounted for on any other than an electrical basis. We know from the experiments in which the action current of one muscle or nerve is made to excite another ("rheoscopic frog" experiments) that these currents have the properties (intensity, duration, rate of change) required for stimulation of the tissue. There are many other instances of transmission of excitatory influence from cell to cell which point to a similar conclusion. Thus adjacent spermatozoa influence one another's rhythm of movement, so that in a group or clump of these cells all are soon found beating in unison (F. R. Lillie, 1913); ciliated cells also transmit excitatory influence, as shown by the waves of accelerated activity passing over the surface of a ciliated epithelium; swimming plates brought within a short distance of one another transmit a similar influence (R. S. Lillie, 1914, p. 428); the same is true of the detached cilia of *Paramoecium* and other Protozoa (Alverdes, 1922). It is difficult to refer the transmission of stimulating effects through the medium in such cases to other than electrical conditions. The transmission of many forms of correlating influence in the growth and development of plants and animals also appears to have an electrical basis.[1]

In inorganic systems the connection between chemical change and electrical change has long been familiar, and is illustrated in the action of batteries and in electrolysis. The transmission of chemical influence to a distance through the intermediary of electric currents depends on this interconnection. Such transmission is independent of the transport of material, and its speed is limited only by that of the electric current (3×10^{10} cm. per sec.). In any electrochemical circuit the rate of chemical change at one electrode is controlled by that at the other in the manner defined by Faraday's law. Hence by changing the chemical conditions at one electrode we may initiate, promote, or retard chemical reactions at another electrode situated at a distance; various examples of this effect were described by Ostwald in 1890 under the name of "chemical distance action."

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[1] See my recent article "Growth in living and non-living systems" (R. S. Lillie, 1922) for a brief discussion. Also R. S. Lillie (1917), pp. 157 ff.

The transmission of chemical influence to a distance in living protoplasm appears to be based upon conditions of essentially the same kind, in which the surfaces of the protoplasmic structures (membranes, films) play a part similar to that of the surfaces of electrodes. A simple inorganic experiment illustrating the transmission of chemical influence along a metallic surface may illustrate the analogy more clearly. When a platinum wire (e.g., 20 cm. long) immersed in dilute H_2SO_4 is touched at one end with a piece of zinc, bubbles of hydrogen instantly appear along its whole length. In this case the transmission of the reducing influence to a distance depends on the formation of an electrical couple in which the zinc is anode and the platinum cathode; hydrogen is accordingly freed at the surface of the platinum wherever the intensity of the current is sufficient.[1] In an analogous manner a local mechanical or other change in an irritable protoplasmic system, e.g., nerve fiber, calls forth chemical change not only at the region immediately affected, but also at a distance, as a result of the electric current which at once arises between altered and unaltered regions; upon this effect the transmission of the excitatory influence depends.

An inorganic system (or "model") showing a type of transmission having many features closely resembling those characteristic of nerve and other rapidly conducting protoplasmic systems is a passive iron wire immersed in dilute nitric acid (R. S. Lillie, 1918, 1919, 1920). In this system the surface of the metal is covered with a thin continuous film of oxide impermeable to the acid, hence the metal remains non-reactive or "passive," so long as it is left undisturbed. If, however, one scrapes the surface with glass, or touches it with ordinary (active) iron, zinc, or similar metal, a reaction accompanied by effervescence and disintegration of the oxide film is at once initiated and sweeps rapidly from end to end of the wire. In this experiment the transmitted effect does not become less as the distance from the activating contact increases (as in the experiment with platinum just described), but is capable of traveling over an indefinite length of wire with undiminished intensity. The reason for this difference is that in the passive wire system the same chemical reaction is renewed or repeated at each successive area of the wire, i.e., the local reaction is self-propagating, like the excitation wave in a muscle or nerve. In acid above a certain strength (55–60 vols. per cent HNO_3 of 1.42 s.g.) the reaction is also self-limiting, i.e., each successive region becomes active, transmits its state of activity to adjoining regions, and then immediately reverts to the passive state. In strong acid the passive state is the state of stable equilibrium; passivity may in this sense be compared to the resting state of an irritable protoplasmic element. Immediately after becoming passive the wire remains for a certain period in a state of imperfect transmissivity in which an activation wave travels for only a limited distance; within a short time it recovers its previous condition and transmits to an indefinite distance as before (R. S. Lillie, 1920).

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[1] Various effects illustrating the influence of chemical distance-action in the formation of precipitation structures from metals immersed in solutions of K-ferricyanide are described in the paper above cited (R. S. Lillie, 1917).

In this system transmission is the result of cathodic reduction of the surface film of oxide under the influence of the local electric current flowing between the active and the passive areas (Fig. 1). Any active area is anodal; the passive areas are cathodal, and are hence subjected to the reducing action of the current. Wherever this current is sufficiently intense, i.e., for a certain distance beyond the boundary between the active and passive areas (*XY* in Fig. 1), the film is reduced to a lower state of oxidation and broken down; the region thus altered becomes at once active, i.e., anodal, and repeats the effect at adjoining regions. Hence whenever any local region is rendered active, e.g., by mechanical disruption of the film, the state of activity is automatically transmitted over the whole surface of the wire. In the automatic repassivation above described, electric factors also enter; any region on becoming active becomes at the same time anodal, and is subjected to an electrochemical oxidizing influence; under these conditions the passivating film is rapidly re-formed.

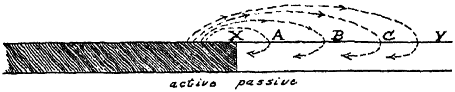

FIG. 1.—Indicating the conditions of the local circuit at the boundary between the active and the passive areas of an iron wire in nitric acid; the direction of the current (positive stream) is indicated by the arrows, the active region (shaded) being anodal, the passive cathodal. The local intensity of the current in the passive region (and hence the reducing or activating effectiveness) decreases in the order $A < B < C$; beyond a certain distance from the boundary, e.g., *XY*, it will be insufficient to activate.

The various parallels between this type of transmission in a protoplasmic system like nerve cannot be described in detail in this chapter, but the chief resemblances may be summarized briefly as follows. (1) Both systems show *polar* activation; the passive wire is activated when it is made the cathode in a circuit (i.e., connected to the Zn of a battery), and is stabilized (or rendered more resistant to activation) when it is made the anode. This behavior resembles that described in irritable tissues under the "law of polar excitation." (2) Altering the state of surface polarization (by passing a current between metal and solution) affects the readiness with which activation is induced (e.g., by mechanical means); if two passive wires (immersed in dilute HNO_3) are connected through a key with a battery (using a current too weak to activate), during the flow of the current the cathodal wire becomes more susceptible to activation, and the anodal wire less susceptible, than when the current is not flowing. There is here a close analogy to the electrotonic changes of excitability in muscle and nerve. (3) Mechanical, chemical, and electrical agents all cause activation in both types of system; the essential change is rapid alteration of the surface film to the degree required to produce a sufficient local current; the resultant reaction has an "all-or-none" character. (4) In electrical activation a slowly increasing current is less effective as an activating condition than a rapidly increasing current, or than the sudden closure of a current of the same final intensity. This feature is highly characteristic of the electric excitation of irritable tissues, as already seen. A similar rule holds for mechanical activation; a sudden blow or pressure stimulates a nerve while a slow one will not; similarly a rapid scrape with glass is required to activate a passive wire. The basis of these resemblances is apparently the automatic tendency of both systems to repair interruptions in the continuity of the film. The alteration must be extensive and sudden, or the change in the film is insufficient to initiate a propagated wave of activation. (5) An activating current must flow for more than a certain time in order to be effective; this time is briefer the more intense the current. (6) Summation effects are highly characteristic; when a passive wire is activated by scraping with glass a rapid succession of scrapes is much more effective than a slow succession; similarly with a rapid succession of brief electric currents. (7) Propagation of the activation wave is associated with, and dependent upon, a local variation of electrical potential due to local alteration of the surface film of oxide. Similarly in nerve and other conducting tissues a variation of potential accompanies and apparently determines transmission, and is associated with a variation of permeability. (8) The speed of propagation of the activation wave in passive iron exhibits a dependence on temperature, and on the electrical conductivity of the surrounding electrolyte solution, resembling that observed with irritable living tissues such as nerve.[1] (9) Immediately after the passage of an activation wave the passive wire transmits activation imperfectly; after an interval it recovers its former properties (analogy to refractory period).

IX. NERVOUS AND OTHER FORMS OF PROTOPLASMIC TRANSMISSION

In certain forms of protoplasmic transmission structural changes in the surface film can be directly observed. This is well illustrated in the response of the *Echinarachnius* egg to insemination. A change involving loss of coherence of the cortical layer of the egg travels from the point of entrance of the sperm to the opposite pole (Just, 1921) (see Sec. VIII); this change is immediately reversed, and within a short time the surface recovers its former properties. Other instances of changes in the plasma membrane during excitation have been described above.[1]

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[1] Results not yet published in detail.

In the transmission of a single excitation wave in a rapidly conducting irritable element like a nerve fiber a temporary change of a similar kind in the surface film, only much more rapid, must be assumed to occur. In other words, as the nerve impulse passes any point in a nerve axone there is a local structural alteration of the surface film, followed immediately by a return of the former state. The bioelectric variation is the index of this structural change; this variation lasts (at 20°) apparently between .001 and .002 second at an excited region of a frog's nerve. The accompanying local bioelectric current is the means by which the local state of excitation is transmitted to the adjacent region; transmission is thus essentially a case of secondary electrical stimulation. The subjoined diagram (Fig. 2) may be regarded as illustrating the essential conditions in a nerve axone the passage of an excitation wave. The analogy to the conditions in the passive iron model will be clear from this diagram. At the region (*BB*) just beyond the active area (*AA*) the current of the local bioelectric current traverses the protoplasmic surface in the direction from protoplasm to medium; this direction, according to the law of polar stimulation, is that required for stimulation. If we assume a sufficiently rapid development of the local action current (0.5–1.0 σ), and a sufficient length of nerve (1.5–3 cm.) stimulated secondarily by this current, the observed rate of transmission (*ca.* 30 m. per sec. at 20°) can be accounted for (R. S. Lillie, 1914). The correlation between the rate of rise of the action current in a conducting tissue and the speed of propagation has already been pointed out.

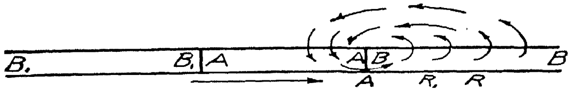

Fig. 2.—In this diagram the area *AA* represents the region of the nerve-axone which is active, or occupied by the excitation-wave, at the instant under consideration; its length in a frog's motor nerve at 20° is about 6 cm. The wave is regarded as moving in the direction of the large arrow. The region now undergoing secondary electrical stimulation by the local action-current flowing between regions *AA* and *BB* extends to the distance *AR* (*ca.* 3 cm.) beyond the wave-front. The direction of the current (positive stream) in part of the local circuit is indicated by the small arrows. The region (B_1B_1) immediately behind the excitation-wave is temporarily in a refractory state.

That electric currents traversing the extracellular media are the chief factors in the spread of excitation is also indicated by the correlation between the electrical conductivity of the medium and the speed of propagation, as observed by Mayor (1917) and Pond (1920).

Transmission in nerve is to be regarded not as a unique or specialized function peculiar to this and similar tissues, but as an example of a type of process occurring everywhere in irritable protoplasm. The special features of the process (its velocity constants, metabolic and other features, etc.) vary widely in different forms of protoplasm; but the general underlying chemical and structural conditions are apparently of the same fundamental nature in all cases. The fundamental condition is the presence of thin polarizable partitions or films, consisting largely of chemically alterable material, and separating chemically dissimilar regions which are at the same time electrical conductors. As the film material changes, under the influence of mechanical, electrical, or other "stimulating" conditions, the electromotor and osmotic properties of the film undergo parallel change with consequent variations of interfacial potentials and the production of local currents. Propagation of such changes may occur if the film material is chemically alterable under the polarizing action of these currents. According to this conception, the essential basis of transmission, as of the other phenomena of reactivity in living protoplasm, is to be found in the polyphasic and film-partitioned character of the protoplasmic system.[1]

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[1] Compare Verworn's description (1913, p. 121) of the conduction of the structural change along the stimulated pseudopodium of *Difflugia.*

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[1] For a more detailed discussion of the conditions of transmission, cf. R. S. Lillie (1923), chap. xv.

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SECTION V

THE PHYSICAL STRUCTURE OF PROTOPLASM AS DETERMINED BY MICRO-DISSECTION AND INJECTION

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THE PHYSICAL STRUCTURE OF PROTOPLASM AS DETERMINED BY MICRO-DISSECTION AND INJECTION

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The existence of a viscid substance within living cells was recognized as early as the latter part of the seventeenth century, when Hooke, Malpighi, and Grew made their discoveries of the cellular structure of plant tissues. More than a hundred years later the observations of Corti and Treviranus on the existence of streaming movements within plant cells led to a recognition of the fact that the cell contents are essentially fluid.

The term "protoplasm" was proposed by von Mohl in 1846 for the "slimy, granular semifluid" constituents of plant cells, which he distinguished from the cell wall, nucleus, and cell sap. It was several years before this, however, that the first recognition of the true living substance, as such, was made by Dujardin, when he proposed the term "sarcode" for the living material of the foraminiferan body and of lower animals in general. Dujardin defined sarcode as a "living jelly, glutinous and transparent, insoluble in water, and capable of contracting into globular masses and of adhering to dissecting needles so that it can be drawn out like mucus."

Soon after this, numerous investigators identified Dujardin's animal sarcode with von Mohl's plant protoplasm as the fundamental life-substance of the cell. Their conclusions, however, were lost sight of because of the more carefully worked-out theory of Schleiden which relegated the seat of vital phenomena to the cell wall. It was not till 1863, through Max Schultze, that the universal occurrence and fundamental similarity of protoplasm in all living beings became generally recognized and protoplasm came to be regarded, as Huxley termed it, the physical basis of life.

When it was once realized that protoplasm is the seat of all life-processes, speculative reasoning cast doubt upon the possibility of a liquid being able to manifest the variety of phenomena peculiar to life. The apparent lack of movement in the majority of animal cells and the frequent detection of structure in protoplasm suggested the existence of a solid substratum. Protoplasm was, therefore, regarded by many as a contractile solid of complicated organization which might contain a fluid in its interstices.

From these ideas arose the reticular and fibrillar theories of protoplasmic structure. Heitzmann, Fromann, and others claimed that protoplasm possesses a delicate reticular structure, while Flemming believed that the substratum of protoplasm is essentially filamentous. These conceptions of a visible structure were greatly strengthened by the general use of fixing agents which coagulate protoplasm. Many, however, who devoted themselves to experimental studies on living protoplasm maintained its essentially fluid nature. Among these may be mentioned Berthold and Bütschli who argued that reticular and filamentous networks in protoplasm can be artifacts. A. Fischer and Hardy then showed that the various structures presumably existing in protoplasm can be produced in egg albumen when it is coagulated with the proper reagents.

Bütschli, from 1876, had always maintained that protoplasm obeys the fundamental laws of a fluid mass. He experimented with oil emulsions and became convinced (1892) that protoplasm possesses an alveolar or foam structure similar to that seen in artificially produced oil lather. He attributed the lack of visible structure in hyaline protoplasm to the extreme attenuation of its alveolar walls (p. 264, 1894 ed.).

Although, from a purely observational point of view, Bütschli's theory stands on no firmer basis than Altmann's granular theory (e.g., granules in hyaline protoplasm may be invisible in the same way as colorless glass beads in oil), it has been the more generally accepted because of its close analogy to our present conceptions regarding the structure of hydrophilic colloids. The great service rendered by Bütschli is in his having firmly established the fact that protoplasm is essentially a fluid.

By the application of newer methods we have since learned that the alveolar structure observed by Bütschli is due, in most cases, to microscopically gross inclusions which may be eliminated without affecting the viability of the protoplasmic matrix. The microscope thus far has revealed no structure within this matrix—its colloidal nature is indicated not so much by its appearance, as by its behavior. On the other hand, protoplasm is a cellular unit which cannot exist without its nucleus and its cortex and, therefore, must be regarded not as a "stuff" but as a mechanism consisting of visibly differentiated and essentially interrelated parts.

I. METHODS

In devising methods for ascertaining the physical nature of protoplasm and of its constituents, account must be taken of the fact that protoplasm exists only in microscopic units of structure. The operations must, therefore, be done in such a way as to enable one to see the results in the field of the compound microscope.

Considerable work has been done in detecting the effects of external agents on the streaming movements which normally occur in the protoplasm of certain cells, but the continuance or cessation of movement does not necessarily imply viscosity changes. Crushing experiments, with the use of a mechanical compressorium, are also open to criticism because there is no way of ascertaining whether the resistance to compression is due to an external pellicle or to the viscosity of the interior of the cell.

A surer method for detecting viscosity changes is that of Němec (1901, 1915), A. Heilbronn (1912, 1914), G. and F. Weber (1916), and others, who used as their criterion the effect of gravity on the dislocation of starch grains in plant cells.

The centrifuge method was used on eggs by Lyon (1907). Morgan (1910), Conklin (1910, 1917), L. V. Heilbrunn (1915, 1920, 1921), and others have used this method to detect localized denser areas and viscosity changes in marine ova Němec (1915) and Weber (1916) among others have used it on plant cells. This method lends itself well to a statistical study of viscosity changes. The objectionable features consist in (1) the rather drastic action of centrifugal force on delicately balanced states of viscosity; and (2) the lapse of time which intervenes between the removal of the cells from the centrifugal action and their observation under the microscope.

The electromagnet method of A. Heilbronn (1922) is more exact but rather limited in its scope. Heilbronn has devised a magnet whose poles are mounted close together on the stage of the microscope. A myxomycete plasmodium containing ingested iron particles (0.05–0.2 mm. in size) is then placed in the field of the microscope between the poles of the electromagnet and the extent of the pull on the iron particles is noted. Seifriz (1924) has recently applied this method to marine ova into which an iron or nickel particle is introduced with a micro-dissection needle.

The physical states of protoplasmic constituents have also been studied by means of the electric phenomenon of cataphoresis. If an electric current be passed through a fluid containing suspended particles, the electric charges of the particles will cause them to travel toward the oppositely charged poles. Presumably this phenomenon may also occur within the fluid protoplasm. However, the unsuitability of this method for the determination of viscosity changes has been pointed out by Bersa and Weber (1922) who have shown that the electric current itself may produce changes in the viscosity of the protoplasm.

A valuable adjunct for studying the physical nature of protoplasm is the detection of Brownian movement by means of dark-field illumination. In ordinary transmitted light a similar oscillatory movement can be detected in particles suspended in a true liquid. It was in this way that Robert Brown himself discovered the movement. Protoplasm is usually too viscid to exhibit such a movement of the ordinarily visible granules. Occasionally, however, it may occur and has been so recorded by many investigators. The dark-field method is far more satisfactory and there are indications that the movement of ultramicroscopic particles rendered visible by this method is more widespread in protoplasm than has generally been recognized. One of the main difficulties attending this method is the presence in most cells of highly refractive granules in such abundance that the field is too brilliantly illuminated for detecting the existence of Brownian movement.

The micro-dissection of cells under the high magnifications of the microscope was first made by Kite (1912), also Kite and Chambers (1912) in animal cells with the use of the mechanical pipette holder and moist chamber devised by Barber and Burrows (Barber, 1907, 1911*a*, 1914). The advantage of Barber's method over that of any other lies principally in Barber's moist chamber. The micro-needles, which are of glass and which may be made either by Barber's or by Chabry's (1887) method, extend from the mechanical holder into a moist chamber on the microscope stage. A cover slip serves as a roof for the moist chamber, and the tips of the needles operate in a hanging drop containing the cells to be dissected, which are pressed against the *undersurface of the cover slip* (Fig. 1). There being no obstacle above the cover slip, oil immersion objectives may be used for observation. For a detailed description of the method as applied to micro-dissection, see Chambers (1918).

Barber's instrument has proved inadequate mainly because of the difficulty in controlling its movements. This is undoubtedly the reason why a great deal of the earlier work in which this instrument was used has since proved to be erroneous.

The dissection work described in this section was mostly done by means of a new instrument built on a principle entirely different from that of Barber's (Chambers, 1921, 1922). Very recently an excellent instrument has also been devised by Péterfi (1923).

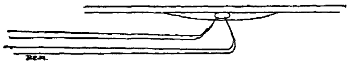
FIG. 1.—Diagram showing method of operating two dissecting needles in a hanging drop suspended from roof of moist chamber.

With all the improvements in this instrument for carrying the micro-needles, there has been no fundamental change in the moist chamber or in the making of the glass needles as first devised by Barber. The tips of the needles taper rapidly to a point of invisibility, and they are usually bent at an angle for operation purposes. A photomicrograph of one is shown in the lower left-hand corner of Figure 18*b* (p. 274), where it may be contrasted with an isolated chromosome which is about 0.002 of a millimeter wide.

The micro-dissection apparatus or micro-manipulator mechanically moves two needles independently of each other in any of the three possible planes. The accuracy of the movement is such that the tip of the needle can be carried evenly across the field of a high-powered objective, and be reversed instantly at the will of the operator. One may appreciate the delicacy of the operation when one realizes that the entire field of a 2 mm. apochromatic objective with a No. 4 compensating ocular is only 0.035 sq. mm. in area.

One of the essential features of this work is proper illumination. Thus far, a specially constructed substage condenser has been used which possesses a working focal distance of 8 mm. and which illuminates about seven-tenths of the back lens of a 2 mm. apochromatic objective.

The micro-injection work is probably one of the most fruitful issues of the micro-manipulation method. Barber's mercury pipette has been superseded by simpler and more efficient devices. In Barber's method, heat was used to produce the expansion necessary for driving the material to be injected. This is unsatisfactory because the injection cannot be instantly checked. Two injection instruments have been recently devised (Taylor, 1920; Chambers, 1922) which depend upon mechanical pressure as a driving force. With these instruments, liquids can be driven through a micropipette with an opening only half a micron (0.0005 mm.) in diameter. The important feature is that the amount of injection can be instantly controlled at any given moment.

In spite of the present degree of perfection of the apparatus, the micro dissection and injection method is open to criticism because it depends so much upon individual and subjective interpretations. However, a great deal of experimental work can be criticized in the same way, and it is to be hoped that with the advent of more workers in this field the few conclusions which have thus far been recorded may serve as an incentive for more extended investigation.[1]

II. THE STRUCTURE AND VISCOSITY OF CYTOPLASM

1. *The cytoplasm of contiguous cells:*

In order to obtain anything like a correct notion of what protoplasm is, we must realize that it exists only within the confines of a cell. In plants the cell is usually separated from its neighbors by a rigid wall of cellulose. On the other hand, animal cells are not as a rule confined within rigid walls. This allows them in most cases to be packed together more closely than is possible in plant cells. It is, however, wrong to consider the typical animal cell as actually naked. If it does not possess an extraneous membrane of some kind, it is usually surrounded by a cement-like substance which may serve to hold contiguous cells together. It is a question whether protoplasmic bridges between contiguous cells, a feature well known in plants, are common in animals.

In plants, cell division usually occurs by the deposition of separate granules which subsequently coalesce to form a wall between the two daughter-cells. Frequently the union of the wall substance is incomplete so that pores persist through which the daughter-cells remain connected by numerous protoplasmic bridges.

The animal cell, on the other hand, divides by an equatorial constriction which cuts the cell in two. Occasionally the division is incomplete so that the two daughter-cells remain connected by a bridge of protoplasm. This method, however, offers no opportunity for the formation of the numerous bridges which have been described as occurring in many tissues. If they exist at all they must form after the cell has divided. It is significant that the furrowing process typical for animal cells is frequently met with in plants in cases where completely separated spore cells are to be produced (Farr, 1918; Sharp, 1921).

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[1] The micro-dissection and injection work described in the following pages was done in hanging drops of sea water for marine ova and lymph or blood serum for germ and somatic cells. Most of the experiments were conducted in the Eli Lilly Research Division at the Marine Biological Laboratory, Woods Hole, and the Weir Mitchell Station, Salisbury Cove.

For a detailed summary of the various methods used to ascertain viscosity changes in protoplasm the reader is referred to Weber (1923).

The passage of an injury from one cell to the other when a protoplasmic bridge connects them is shown in the following experiment on chick mesenchyme tissue culture cells. Figure 2*a* shows two daughter-cells half an hour after the commencement of division. Note that the two cells are still connected by a slender strand of cytoplasm. One cell was injured by being torn with a needle, whereupon its nucleus immediately coagulated. After several seconds the effect of the injury became apparent in the other daughter-cell by the coagulation of its nucleus.

In contrast to this is another case where two interkinetic cells were so closely contiguous that the boundary between them could not be seen (Fig. 2*b*). One of the cells was injured by puncturing. Both cells immediately reacted by partially withdrawing from each other, but only the nucleus of the injured cell coagulated, while the other remained normal and alive.

From this it is evident that the effect of a mechanical injury can travel from one cell to another only when there is protoplasmic continuity between them.

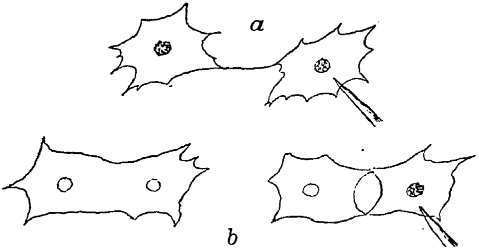

FIG. 2.—Mesenchyme cells in tissue culture. (*a*) Two daughter-cells still connected by a bridge which transmits injury from one cell to the other. (*b*) Two contiguous cells, one of which only is injured by pricking.

It has been claimed that protoplasmic bridges exist between the blastomeres of segmenting echinoderm eggs (Andrews, 1897). This is, however, improbable because, with the needle, the blastomeres can be gently pushed apart or made to roll over one another within the investing egg membrane. The blastomeres have perfectly smooth contours, and they give no evidence whatever of connecting strands. If the drop in which the eggs are being examined be allowed to evaporate, injury sets in and elevations appear on the surfaces of the blastomeres. These elevations grow out as slender filaments which soon produce the effect of bridges extending across the gap between the shrunken and moribund cells (cf. p. 273). The existence of protoplasmic bridges has also been maintained in many other cells, especially in stratified epithelium. They are always figured as fine striations extending across a narrow space between contiguous cells. These striations may be either artifacts or indications of a fibrous structure of the intercellular cement or a combination of both.

In the majority of the cell groups in the metazoan body, there is no evidence whatever for the existence of actual protoplasmic bridges between the individual cells. It is highly probable that protoplasm exists as a morphological and physiological unit in each cell of the body. Some of its functions may be more highly specialized in one group of cells than in another, and the secretions of one group of cells may profoundly affect another; but as regards the fundamental vital phenomena, each cell lives out its own existence.

There are at least two visible structures in protoplasm which are essential for its existence. These are the nucleus and the protoplasmic surface film. If this surface film be destroyed, the protoplasm disintegrates unless a new film can be quickly constructed. In some cells, moreover, the differentiation between the internal and cortical protoplasm has gone so far that the internal protoplasm, even with a surface film, cannot live without its original cortex.

The necessity of the nucleus for the life of the protoplasm of a cell has been so well recognized that the term "protoplasm" is generally applied to an organized system consisting of a differentiated nucleus contained within a mass of cytoplasm. To this definition we must add that the protoplasm in order to exist must be surrounded by a differentiated protoplasmic surface film.

2. *The visible structure of cytoplasm:*

The cytoplasm is a colorless, translucent substance in which there may or may not be imbedded granules and vacuoles in varying numbers. When pigmented, the color is apparently restricted to structural elements (pigment granules) or is in solution in the contents of vacuoles. In blood cells the hemoglobin color appears to be uniformly distributed throughout the cell. The translucency of protoplasm varies with the shape and number of its granules and vacuoles, the matrix or hyaloplasm being transparent.

The presence of visible granules, fibrils, and vacuoles in the hyaline matrix is such a universal feature that most of our conceptions as to the visible structure of protoplasm have been based on the assumption that these inclusions form an integral part of protoplasmic structure. However, in view of the fact that these structures may vary in different cells, not only in form, but also in number, and may be entirely absent in some cells or appear only at different stages in the life of a cell, we must regard them rather as specialized differentiations.

Granular amoebae frequently protrude pseudopodia which are entirely free from granules. These pseudopodia can be cut off from the parent body, and still maintain their integrity. They are irritable, are capable of ingesting food, and can move about in the typical amoeboid manner. Although lacking the visible granules of the parent body, they must still be regarded as masses of viable protoplasm. The sea-urchin egg is another case in point. These eggs in the unfertilized condition can be centrifuged so that all the visible granules except the oil globules are thrown to one side of the egg. If the centrifuging process be carried to an extreme, the eggs are pulled out into cylinders with the granular part at one end. These eggs can be cut with the needle into a granular and a hyaline portion. Upon insemination both pieces are fertilized and both undergo cleavage. The cytoplasm of the egg is, therefore, similar to that of the amoeba in being normally crowded with visible granules which may be gotten rid of without impairment (cf. F. R. Lillie, 1906; Mathews, 1906, 1907).

It is a question whether it is possible to resolve structure in the hyaloplasm. It is claimed that this has been done by ultra-violet light photography. This method, however, is open to the criticism that the ultra-violet rays have a distinctly coagulative effect on protoplasm. The fact that an amoeba, for example, is alive after a short exposure to the rays does not prove that some coagulation has not taken place, for it is possible to coagulate localized areas in the amoeba without irreparably injuring the animal as a whole (cf. p. 263).

Various dark-field investigators (Gaidukov, 1910; Mott, 1912; Price, 1914) find that the cytoplasm is optically heterogeneous whereas the nucleus shows no structure. However, all these investigators examined cytoplasm which still contained inclusions already visible with ordinary illumination. They were not making a critical study of the hyaloplasm itself.[1]

Of the various kinds of visible granules to be found in the cytoplasm there is one, the microsome, which seems to be almost universally present. It is the smallest of the granules, being somewhat less than .2 micron in size but is plainly visible owing to its high refrangibility. In spite of its small size it can, by means of the centrifuge, be thrown out of suspension from the fluid protoplasm of mature echinoderm egg cells. In the dark field it gives rise to diffraction disks, and is of great value for the detection of low viscosity as it readily exhibits Brownian movement when the cytoplasm in which it is suspended is sufficiently fluid.

The other types of cell inclusions vary greatly in different cells and many of them vary in the same cell at different times.

The echinoderm egg is a good example of protoplasm crowded with granules and vacuoles of various kinds. Aside from the microsomes, the most prominent are the macrosomes or alveolar spheres (Wilson, 1899), more or less irregularly shaped bodies from 1–2 micra in diameter. They are closely packed together and their index of refraction is so close to that of the hyaloplasm in which they lie that their presence is betrayed mainly by the arrangement of the microsomes surrounding them. We know little regarding the function of these macrosomes. They are possibly nutritive, for they accumulate in the growing egg (Wilson, 1899) and gradually disappear in the cells of the developing embryo. They are very susceptible to injury and quickly swell and run together when the egg cytolyzes, which suggests that they are quite fluid (cf. Mathews, 1906). In the presence of neutral red, many of them take on a rose-red color. In naturally pigmented *Arbacia* eggs, the pigment is localized in bodies closely resembling the macrosomes. In addition to the macrosomes and microsomes there are minute globules, possibly fatty in nature, distributed through the cytoplasm, and rodlike mitochondria which stain specifically with Janus green and are collected principally in the cortex of the egg.

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[1] With the ordinary dark-field illumination, the hyaloplasm of the amoeba and of the sea-urchin egg shows no structure whatever when freed of its visible granules by centrifuging. This indicates that suspended particles present must be either less than 10 millimicra (M) in diameter, the limit of visibility for the usual dark-field equipment, or they may possess the same index of refraction as the environing medium (Chambers, 1923*b*).

By subjecting sea-urchin eggs to centrifugal force the visible protoplasmic constituents are thrown into four clearly defined zones (Lyon, 1907). The pigment granules occupy a narrow zone (Zone 1) at the periphery on one side of the egg, and the oil globules collect as a so-called oil cap (Zone 4) at the periphery on the opposite side. Next to the pigment zone within the egg is a broad zone (Zone 2) of closely packed macro- and microsomes interspersed with the mitochondria. Between this zone and the oil cap is a transparent zone (Zone 3) of hyaloplasm. The nucleus occupies a region in the hyaloplasm zone close to the oil cap.

On being returned to a bowl of sea water the centrifuged eggs orient themselves with the oil cap uppermost, indicating that the pigment and granular zones lie at the heavier pole. If the eggs be torn with a needle the hyaline zone (Zone 3) is found to be essentially liquid, while the granular zone (Zone 4) is very viscid. This is also indicated by the fact that the hyaline zone shrinks the most when the egg is placed in a hypertonic solution. By dark-field illumination the hyaline zone is optically empty except for the spherical nucleus, the contour of which appears as a thin, bright line. At the boundary between the hyaline and granular zones a Brownian movement of the microsomes is distinctly perceptible, and this furnishes ideal material for a study of the effects of various reagents on Brownian movement deep in the egg and, by inference, on its internal viscosity (cf. p. 297).

We thus have in the sea-urchin egg cell a wealth of cytoplasmic inclusions in the form of microsomes, macrosomes, pigmented macrosomes, oil globules, and mitochondria, all of which are crowded together in such numbers as to mask almost entirely the fluid hyaloplasmic matrix in which they lie.

In the cells of the somatic tissues the hyaloplasm is often quite conspicuous owing to the sparseness of visible granules. It is very evident in cells growing in tissue culture.

The cytoplasmic inclusions also vary considerably with the functional differentiation of the cell. In plant cells the cytoplasm is characterized by the presence of highly specialized bodies, the plastids, which are known to be concerned with the synthetic functions of the plant cell. Plastids are also to be found in certain Protozoa.

3. *The viscosity of cytoplasm:*

The existence of movement in protoplasm has been recorded almost from the earliest days of the invention of the compound microscope. And the active streaming movement, termed cyclosis, in the protoplasm of many plant cells and Protozoa has baffled everyone who has attempted to explain it in physical terms (cf. Kühne, 1864; Bütschli, 1892; Ewart, 1902).

In the protoplasm of metazoan cells a continual translational as opposed to Brownian movement is much less perceptible but nevertheless exists. Gardiner (1895), for example, was much impressed as he observed the incessant flux of all the granules in the otherwise quiescent egg of *Polychoerus*. Similar cytoplasmic movements can be seen, not only in the living cells of the pancreas, nerve, epithelium, etc., but also in the highly viscid cytoplasm in the aster of the fertilized egg (Chambers, 1917*b*).

It is extremely difficult if not impossible to speak at present of the viscosity of protoplasm except in relative terms. Owing to the microscopic dimensions in which protoplasm exists we have as yet no means of securing any absolute value of its viscosity. Seifriz (1920) has made some conjectural analogies by comparing the viscosity of the interior of a sea-urchin egg to glycerine and that of the cortex to bread dough.

The protoplasmic viscosity of different cells varies greatly—the annelid egg, for example, is almost liquid, while the sea-urchin egg is decidedly viscous. The motor nerve cells of the frog and of the lobster are highly viscid masses of protoplasm which can be torn only with difficulty by means of the microdissection needle. On the other hand, the pancreas cell often becomes completely dissipated with a mere puncture of the needle.

The viscid nature of the cortex of most cells is shown by the fact that it can be drawn out into strands with the needle. When the strand is released it gradually withdraws into the cell. The interior cytoplasm is usually much more fluid as is also indicated by the spherical shape of the contained vacuoles. A droplet of olive oil, 5–6 micra in diameter, when injected into the cytoplasm of a mature, unfertilized sand-dollar egg (0.1–0.12 mm. in diameter), immediately assumes the shape of a sphere. If the cortex of a sand-dollar egg be torn, the interior will flow out and immediately assume the shape of a sphere.[1]

4. *The existence of Brownian movement:*

The term Brownian movement has been rather loosely applied to the oscillatory as opposed to the translational movement of cytoplasmic granules, somewhat less than 1–2 micra in diameter, when viewed with ordinary transmitted light. Such movements have been occasionally recorded in protoplasm. Kühne (1864) observed restricted oscillatory movements in the cytoplasm of the amoeba in its normal condition, but he was positive about distinguishing them from what he called the *Molekularbewegung* typical of purely physical suspensions.

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[1] Recently Seifriz (personal communication) has confirmed this difference between the cortex and interior of the sand-dollar egg by observing the travel of a nickel particle which he inserted into an egg and brought under the influence of an electromagnet. The nickel particle was carried through the interior and came to a stop at an appreciable distance from the surface of the egg. If the particle be inserted just under the surface of the egg, the elasticity of the cortical substance was demonstrated by the fact that although the particle could be made to travel a short distance it returned to its original position when the electric current was shut off.

The ultramicroscope is rather unsuited for the examination of protoplasm. On the other hand, dark-ground or dark-field illumination obtained by parabolic and cardioid condensers has been used with considerable success.

The cytoplasm of a cell is usually filled with granules and therefore presents a brilliantly illuminated picture in the dark field. In non-dividing epithelial cells of animals no Brownian movement has yet been detected. Marinesco (1912), in an extended study on nerve cells, found none, and recently Schmitt and I have looked in vain for such movement in the various epithelial cells of the frog and the skate. Apparently the viscosity of these somatic cells is too high (cf. Chambers, 1915) to permit of such movement. In plant cells, however, the presence of Brownian movement has been frequently detected (Chifflot and Gautier, 1905; Gaidukov, 1910; Leblond, 1919; Bayliss, 1920) and also in animal tissue culture cells (Lewis, 1923). The movement may appear occasionally during certain phases of the life-cycle of the cell and sometimes only in localized regions of the cytoplasm.

Owing to the crowded condition of the hyaline macrosomal (2–3 micra in diameter) and the highly refractive microsomal (.1–.3 micra) granules, the egg cytoplasm is ordinarily so brilliantly illuminated in the dark field as to mask the existence of Brownian movements. If, however, the egg be slightly compressed, a distinct but rather restricted shimmering movement can be easily detected in the periphery of the egg. When one's eye becomes accustomed to the light, the existence of a similar movement throughout the entire mass of the egg cytoplasm becomes gradually appreciable. The shimmering particles are neither the hitherto recorded macrosomes nor microsomes, but apparently truly ultramicroscopic particles. The macrosomes and microsomes do not appear to exhibit this movement in the normal unfertilized egg.[1]

When these eggs are submitted to centrifugal action a more fluid hyaline portion collects in a zone between a heavy, densely granular area and the very light oil cap (cf. Lyon, 1907). In the dark field the hyaline zone is optically empty, but along the border of the hyaline and granular zones a distinct but restricted Brownian movement of both the ultramicroscopic and larger granules is easily discernible. If the egg be allowed to stand for several hours in sea water the granules gradually invade and fill up the hyaline zone. The migration of the granules is apparently brought about by the purely physical Brownian movement. The shimmering movement of the granules along the border of the granular and hyaline zones is discernible also with ordinary transmitted light.

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[1] It might be mentioned that the dark-ground illumination is most illusive with respect to the actual shape of the illuminated granules. Whereas with ordinary transmitted light the macrosomes are seen to be more or less irregularly ovoid bodies, they appear spherical in the dark field.

Cells, the cytoplasm of which exhibits no structure in the dark field, are the male germ cells of certain insects. Except for the mitochondria (cf. p. 274), which are definitely localized about the nucleus, the cytoplasm is transparent and optically empty.

5. *Viscosity changes in cytoplasm:*

Most of the earlier investigators attempted to explain protoplasmic movement by assuming that protoplasm consists of a contractile solid with intervening fluid parts. Others concluded that protoplasm is contractile throughout its whole substance. De Bary (1864) in his investigations on the plasmodia of Myxomycetes was convinced that protoplasm is a single substance whose physical character is frequently liable to both local and general variations. It is remarkable that De Bary arrived at his conclusions only a few years before the death of Thomas Graham whose researches on the colloidal nature of matter have since been so fruitful in their application to the physical nature of protoplasm. Bütschli and Rhumbler, in several writings dating from 1891 and 1896, have offered explanations for viscosity changes and protoplasmic movement on the basis of changes similar to those which obtain in inorganic emulsions.

Speculative reasoning, with little or no experimental work, has, however, for long been indulged in regarding the physical nature of protoplasm, and it was not till comparatively recently that investigators have even attempted an exhaustive experimental study of one of its most important phases, viz., its viscosity.

Probably the first to suggest the existence of viscosity changes was von Mohl (1846), who stated that plant protoplasm increases in viscosity with age. More recent investigators who have noted viscosity changes during the life-cycle of the cell are Chifflot and Gautier (1905) and Leblond (1919) in *Algae* (by dark-field observations on Brownian movement), and Peebles (1912) in *Paramoecium* (by cutting experiments). They concluded that these organisms are more fluid just prior to and throughout their reproductive activity than during their vegetative periods.

Not only may the protoplasm of a cell exhibit viscosity changes as a whole but it is of significance that such changes may also occur in localized areas within the cell. Gaidukov (1910) and Price (1914) have recorded plant cells where a localized Brownian movement of granules may suddenly start up in an area previously motionless.

In this connection must also be mentioned the changes in viscosity which occur during the maturation and fertilization of the egg. Albrecht (1898) by crushing echinoderm eggs concluded that an increase in viscosity follows the fertilization process. It is probable, however, that he came to this conclusion not from any direct evidence that the cytoplasm had stiffened but from the toughening of the investing fertilization membrane which results upon insemination. Heilbrunn (1915, 1920, 1921), with the centrifuge method, has definitely proved that the internal viscosity increases after fertilization. He also states (1921) that the viscosity of the sea-urchin (*Arbacia*) and *Cumingia* egg suddenly decreases shortly before cleavage to rise again after the cleavage process is completed. Ödquist (1922) by using the same method found that the viscosity of the frog's egg gradually decreases after fertilization to reach its maximum shortly before the first cleavage. After each cleavage the viscosity rises again. Zimmerman (1923) likewise was able to detect viscosity changes in the segmenting cells of the *Sphacellaria* plant. In the prophase he found an increase in viscosity of the cytoplasm, and in the metaphase, a rapid decrease followed by a gradual increase.

The results of micro-dissection agree with those of Heilbrunn regarding the increase in viscosity subsequent to fertilization of the egg. (For results obtained on the dividing cell, see p. 291.)

6. *Experimentally induced changes in the viscosity of cytoplasm:*

Both M. Schultze (1863) and Kühne (1864) tried the effect of a variety of agents on streaming protoplasm. Schultze found that an increase in temperature, within certain limits, accelerates the streaming, and Kühne found the same for sudden changes of temperature in either direction. He concluded that this is an irritability effect. Kühne found that the movements cease when streaming protoplasm is exposed to induction currents, CO_2, H_2 vapor, ether, chloroform, extremes of temperature, or inclosure in a layer of an indifferent oil. A return to normal surroundings started the streaming again. Kühne ascribed these results to the lack of oxygen (cf. opposing views of Ewart, 1902).

These earlier writers remark on certain changes in protoplasm, which they refer to vaguely as condensation effects. It remained for comparatively recent workers to study the reversible viscosity changes which can be experimentally produced in living protoplasm. Some of the agencies used are temperature changes, dilutions of narcotics, acids and alkalies, hyper- and hypotonic solutions, etc.

a) HYPER- AND HYPOTONIC SOLUTIONS

These produce their effects mainly, at least, by changing the water content of the cell, a hypertonic solution causing the cell to shrink in size and to increase in viscosity (Heilbrunn, 1915, 1920), a hypotonic solution inducing swelling with a corresponding decrease in viscosity.

b) VARIATION IN TEMPERATURE

Extreme cold apparently has the same effect of increasing viscosity with an extraction of water. Greeley (1901, 1903) showed that the infusorian, *Stentor*, shrinks in size and its cytoplasm stiffens at a temperature of 2° C. It returns to normal again when brought back to room temperature. G. F. Weber (1916) and Weber and Hohenegger (1923) also found that low temperatures increase the viscosity of plant cells. On the other hand, L. V. Heilbrunn (1920*b*) by the centrifuge method detected only a lowered viscosity in *Arbacia* eggs at 3° C. (personal communication). Below this the viscosity tends to increase and, if the sea-water containing the eggs be allowed to freeze, the egg cytoplasm coagulates. A. Heilbronn (1922) used his electromagnet method in studying the effect of temperature upon the plasmodium of Myxomycetes. He found that a slight drop below (12° C.) or a slight rise above (33° C.) the normal temperature diminishes the protoplasmic viscosity of the plasmodium of *Reticularia.* This is followed by a slight reaction at both temperature extremes which raises the protoplasmic viscosity.

c) ETHER

Ether produces viscosity changes in protoplasm apparently without affecting its volume.

A. Heilbronn (1914, 1922) found that low concentrations diminish and high concentrations increase the viscosity of the myxomycete plasmodia. These effects are reversible, i.e., the viscosity returns to normal when the organism is removed from the ether. Weber at first (1921) records similar results with *Spirogyra* cells, but later (1922) seems to have secured only an increase in viscosity in the epicotylar cells of *Phaseolus.* A. Heilbronn (1922) regards the liquefying action of low concentrations on the plasmodia of Myxomycetes, as irritation effects which accelerate amoeboid activity.

Both A. Heilbronn and Weber agree with the coagulation theory of narcosis. On the other hand, L. Heilbrunn (1920) claims that the reversible effect of 2.5 per cent ether on the sea-urchin egg occurs only when the viscosity is diminished. With higher concentrations of ether (3 per cent+) the increased viscosity, according to him, is irreversible. He therefore concludes that narcosis implies a diminution in viscosity of the protoplasm. My results with the micro-dissection method and by observing Brownian movement in the dark field do not agree with this. In 2 per cent ether, Brownian movement was slowed down but did not cease, and cleavage was delayed but not stopped. In 2½ per cent ether, cleavage was stopped and, both by means of the needle and by the cessation of Brownian movement, this was shown to be accompanied by a decided increase in viscosity (cf. p. 300).

It is of interest to note that the cessation of Brownian movement caused by a narcotic dose of ether occurs throughout the entire egg protoplasm and not only at its surface. As already described (p. 245), one can observe in the centrifuged sea-urchin egg a distinct but restricted Brownian movement of ultramicroscopic particles at the boundary between the granular and non-granular layers into which the visible cell constituents have been thrown. When eggs so treated are placed in sea water containing 2.5 per cent ether, the Brownian movement slows down and ceases within a minute. The slowing effect occurs simultaneously all through the egg. The eggs can withstand complete cessation of the Brownian movement by ether for only five minutes or so, after which an irreversible coagulation occurs with death of the cell.

An interesting feature is involved in the action of ether on eggs swollen in hypotonic sea water (50 parts tap water+50 parts sea water), which causes the Brownian movement to become excessive. Concentrations of ether too low to stop Brownian movement in normal eggs will stop it in hypotonic-treated eggs. For example, 1 per cent ether which has no effect whatever on normal eggs will stop the movement in the hypotonic-treated eggs within 20 minutes. This effect is reversible because the eggs, on being returned to pure sea water, shrink to their normal size, exhibit Brownian movement again, and continue their development. Apparently the susceptibility of protoplasm to ether narcosis increases with the degree of dilution with water.

d) CARBONIC ACID

The effect of carbonic acid on viscosity is similar to that of ether. By using the centrifuge Jacobs (1922) found that the protoplasm of certain Infusoria is distinctly liquefied in low concentrations of CO_2 water, whereas it is stiffened in higher concentrations. On removal from the CO_2 water the Infusoria quickly regain their original consistency.

e) MECHANICAL AGITATION

The viscosity of a cell can be diminished by inserting a needle into the cell and mechanically agitating the cytoplasm. This phenomenon is fully discussed in the subsection on the aster in the fertilized egg (cf. pp. 288 and 291).

f) OTHER AGENTS

Alkalies tend to diminish, whereas acids increase the viscosity of amoeba cytoplasm without appreciable injury (Chambers, 1921).

Sublethal concentrations of certain saponins (*Quillaja saponin* and *digitonin*) diminish the viscosity of echinoderm egg cells (Page, Chambers, and Clowes, in press).

An electric current of a certain low intensity has been found by Bersa and Weber (1922) to produce a reversible increase in the viscosity of plant protoplasm.

III. THE EXISTENCE IN PROTOPLASM OF A DIFFERENTIATED CORTEX AND INTERIOR

An extraordinary feature of protoplasm is the fact that, although it consists largely of substances which are soluble or are suspended in water, nevertheless it maintains itself perfectly within an aqueous environment. The question is whether its maintenance depends upon the existence of a differentiated surface or whether protoplasm may be regarded as a substance whose essential properties exist throughout its entire mass.

The existence of a differentiated outer layer on the fluid protoplasm of a cell was probably first suggested by Pringsheim. This was considered by Kühne (1864) as analogous to that which forms on the surface of a drop of egg albumen in distilled water. He observed that if the protozoan ciliate, *Stentor*, be crushed, the fluid protoplasm contents of the interior will pour out in a stream whose borders quickly become surrounded with a membraneous covering, which he suggested is due to the precipitation of an albuminous substance.

Quincke laid stress on the immiscibility of protoplasm in water and concluded that the surface of protoplasm must have properties of a fatty nature. This idea led to Overton's (1900) lipoid theory of the cell membrane.

It was Pfeffer (1890), however, who gave significance to the cell membrane as being responsible for the semi-permeable properties of protoplasm. In his plasma membrane theory, he assumes the surface of protoplasm to be an osmotic film one or two molecules thick, and consisting mainly of proteins. In order to demonstrate the existence of a semi-permeable plasma membrane, he performed the following experiment. By immersing a living cell in a weak solution of HCl he was able to cause the plasma membrane to assume the condition of rigor without destroying its semi-permeability. Under these conditions a dye which does not penetrate the surface membrane will enter through a tear and rapidly diffuse throughout the interior of the cell.

Pfeffer also repeated Kühne's experiment by crushing *Vaucheria* and *Hydrocharis* filaments, and he showed that the extruded spheres of protoplasm possess the same osmotic properties as of the original cell.

The importance attached to the plasma membrane in the vital activities of the cell gave support to its being considered as a definite cell organ. Just as Virchow had announced the dictum *omnis cellula e cellula* and Flemming *omnis nucleus e nucleo*, so De Vries claimed that all plasma membranes originate from a previous membrane. Pfeffer, however, was able to show that the formation of surface films is an innate property of any part of the protoplasm and can be readily produced *de novo*. One of his experiments consisted in introducing asparagin and gypsum crystals into the body of a myxomycete plasmodium. The crystals, imbedded in the protoplasm, gradually imbibed water and formed vacuoles bounded by distinct surface films. More recently Kite (1915) produced artificial vacuoles within the cell by means of micro-injection. Aqueous solutions injected into protoplasm may or may not produce vacuoles because the formation of a bounding film depends upon the solution injected and the way it is injected (Chambers, 1917*a;* cf. p. 264).

The readiness with which surface films in protoplasm can be formed only to disappear again by fusion of the protoplasm is strikingly shown in the plasmodium of the Myxomycetes, *Badhamia*, which can flow through wet cotton wool. Lister (1888) used this method to filter out the contained spores in order to obtain clear protoplasm. Indeed, the plasmodium normally creeps through and permeates rotting wood, coming to the surface only just prior to sporulation.

In order to obtain a proper conception of the nature of a true protoplasmic film, one must realize that the protoplasm of most cells also possesses a more or less solid cortex of an appreciable thickness. In addition to this, many cells are either completely or partially invested in extraneous membranes or pellicles which are probably products of secretion or precipitation (cf. Seifriz, 1921).

1. *The existence of an extraneous pellicle:*

It appears to be generally true that cells which normally exist by themselves possess a membrane or pellicle external to the true protoplasmic surface of the cell. For example, in the protozoan ciliates, *Paramoecium* and *Stentor*, this pellicle can be seen on blister-like elevations which occur when these organisms are placed in an abnormal environment. Mere compression will often produce these blisters. Pricking the blister with a micro-needle causes it at once to collapse. In some Protozoa, e.g., *Vorticella* and *Amoeba verrucosa*, the pellicle is tough and difficult to tear with the needle (cf. p. 279). Even in the very fluid amoebae, an extensible but distinctly extraneous pellicle can be demonstrated (cf. Greef, 1892).

The pellicle may be a secretion product and probably serves as a mechanical support. The significance of the pellicle of the *Amoeba* is discussed in its relation to amoeboid movement (p. 280). In addition to the pellicle, many Protozoa secrete a slime. It is by means of this adhesive slime that the amoeba often fastens itself to its substratum. In an amoeba which has been stationary for some time the amount of slime excreted may be considerable. The extensibility of this slime is such that if the amoeba be dragged a distance through the water and then released the amoeba will be passively drawn back almost to its original position. It is also possible that slime formation has something to do with the structure of the extraordinary undulating membranes in certain ciliates. This is shown in the *Blepharisma* (Chambers and Dawson, in press). The undulating membrane, on being touched with the micro-needle, breaks up into cilia which beat separately and out of unison so long as the needle touches them. When the needle is removed the cilia fall into line again as a fusion takes place which spreads from their bases to their tips until, within a second or two, the whole row of cilia appears again as a structureless, undulating membrane. This may be explained by the secretion of a slime which spreads over the cilia and joins them together into a homogeneous-appearing sheet.

The early ovarian egg possesses no appreciable pellicle, but as the egg grows in size preparatory to being extruded into the sea water the existence of a pellicle becomes more and more pronounced. On fertilization it is this membrane which is lifted off as the fertilization membrane. Shortly after the elevation of this membrane a new pellicle forms on the surface of the egg which, in the echinoderm eggs at least, serves to hold the blastomeres together during their development. In the sand-dollar and sea-urchin eggs this pellicle is especially well pronounced, and has been regarded by some as an integral part of the protoplasm, an ectoplasm, the so-called hyaline plasma layer (O. Hertwig, 1876; Hammar, 1897; Ziegler, 1898; McClendon, 1911; Painter, 1918; and Just, 1922). Herbst (1900) found, however, that this pellicle does not form in calcium-free sea water, and the blastomeres fall apart. On being returned to normal sea water each blastomere develops a fresh pellicle which holds the subsequent blastomeres together. Goldschmidt and Popoff (1908) and Loeb (1909) also regarded this pellicle as a binding membrane for the blastomeres. By simply tearing the pellicle with a micro-needle, the blastomeres fall apart (Chambers, 1923*a*). It is of interest to note that the pellicle forms only on that surface of the blastomeres which is in direct contact with sea water and is presumably a precipitation product. Elsewhere, the blastomeres are free to slip over one another if disturbed with the needle.

The persistence of this pellicle as a tough, structureless investment has been followed through the gastrula to the late pluteus stage of the sand-dollar egg. In order to isolate an epithelial cell of the pluteus, it is necessary to tear the pellicle, whereupon the cells in the torn area round up and can be slipped out through the tear (Fig. 3). The invaginated endoderm is likewise covered by a pellicle continuous with the one on the exterior of the larva.

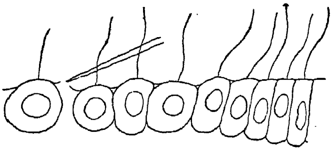
FIG. 3.—Effect of tearing the pellicle overlying epithelium of sand-dollar gastrula.

In the sand-dollar pluteus we have an example of a young metazoan body, and it is significant that all the cells, both ectoderm and endoderm, which are in contact with the environment of the animal are covered on their exposed surfaces by a continuous, structureless membrane. The so-called cuticular border which is a continuous membrane lining the respiratory and alimentary canals in the higher Metazoa may be analogous to the pellicle of the larval echinoderm. The ciliated epithelium in the mouth of the frog, for example, can be torn off in strips which, on tearing with the micro-dissection needle, are found to be held together mainly by a structureless cuticular border continuous over their originally free surfaces.

2. *The cortex and interior of protoplasm:*

a) PHYSICAL DIFFERENCES BETWEEN THE CORTEX AND INTERIOR

A feature of the protoplasm of many cells is the existence of a more or less differentiated cortex. When the cortex is well differentiated, it is an appreciable zone of protoplasm which is more solid than the interior. This is specially well developed in many Protozoa. *Paramoecium*, for example, possesses a firm cortex, the ectoplasm, of a uniform thickness over the body of the organism. It is a structural modification which cannot be easily, if at all, repaired by the more fluid endoplasm. In *Paramoecium* it is an elastic jelly-like wall which is under a certain tension due to internal turgor. This can be shown by tearing the ectoplasm with a micro-needle. The fluid interior then pours out, while the torn edges of the ectoplasm curl in, and the entire animal shrinks in size. If the edges of the tear meet they unite and further outflow of the endoplasm ceases, otherwise all the endoplasm flows out and the ectoplasm soon disintegrates. The endoplasm as it pours out into the surrounding water occasionally forms a delicate surface film which maintains the integrity of the extruded mass. Usually, however, the endoplasm becomes entirely dissipated and disintegrates. In some species, e.g., *Paramoecium bursaria*, the torn surface very readily forms a surface film which frequently persists. The integrity of the cell is thereby maintained. In the few experiments that have been made on this form, the exposed endoplasmic surface film is apparently unable to regenerate a differentiated ectoplasmic layer. Complete recovery probably occurs by a gradual contraction of the animal until the ectoplasmic edges, bordering the endoplasmic surface film, meet and unite.

In the somatic cells of the Metazoa and of plants, the existence of a differentiated cortex of appreciable thickness has not yet been determined. Somatic metazoan cells which have so far been dissected either possess a delicate protoplasmic membrane (cf. p. 257) or are glutinous and jelly-like throughout with no appreciable difference between the cortex and the interior.

The results of micro-dissection prove the presence of a viscid cortical layer in the unfertilized mature eggs of some echinoderms, especially the starfish. The extraneous egg membrane must first be removed. The cortex may then be seized with a micro-needle and pulled out into long strands which, on being released, slowly draw back into the egg. If the cortex be torn through and the egg brought under compression the more fluid interior flows out, on the surface of which a film quickly appears. This new surface is more fluid than the original cortex, for a strand pulled out from it will rapidly flow back when released. In the echinoderm egg there is no visible difference between the cortex and the interior. In an egg which has been torn, one also cannot distinguish the new surface from the original cortex except by their reactions to the needle.

There is evidence for believing that the viscosity of the cortex can be diminished by agitation with the needle and also by the treatment of certain reagents. However, we have no good evidence as yet regarding the regenerative property of the cortex. It is also probable that a diminution in viscosity does occur as a result of fertilization of the egg.

b) PHYSIOLOGICAL DIFFERENCES BETWEEN CORTEX AND INTERIOR

It has also been found that important physiological differences may exist between the cortex and interior of the egg cell (Chambers, 1921*a* and *b*). The following experiment shows this for the starfish egg. If the surface of the mature egg be torn with a needle, and the egg then caught at the opposite side and pulled to the edge of the hanging drop, the compression on the egg produced by the shallow water at the edge of the drop will cause the fluid interior to ooze out through the tear, forming a perfect sphere (Fig. 4). One may so manipulate the process as to cause the egg nucleus either to remain behind in the cortex (cortical remnant) or to pass into the extruded sphere (endoplasmic sphere).

The cortical remnant is relatively solid and tends to remain within the egg membrane. If left long enough it will eventually round up so as to present the appearance of a diminutive egg surrounded by a collapsed and wrinkled egg membrane. On the other hand, the material which has escaped from the interior of the egg into the sea water is fluid and tends immediately to round up.

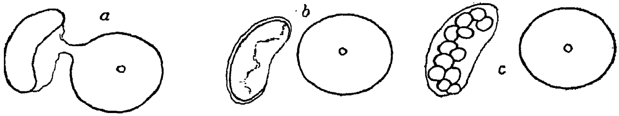

FIG. 4.—(*a*) Production of an endoplasmic exovate by crushing starfish egg. (*b*) and (*c*) Effect of inseminating the ecto- and endoplasmic fragments (Chambers, 1921*e*).

The cortical remnant is readily fertilizable and undergoes normal segmentation, whereas the material which has escaped from the interior of the egg, whether nucleated or not, cannot be fertilized. As long as it possesses an intact surface it appears exactly like a normal egg, and will undergo disintegrative changes similar to those of entire eggs when torn with the needle. If even a small part of the original cortex is cut off with this endoplasmic sphere, the sphere is fertilizable, and the more cortical material present, the more nearly will the sphere approach normal cleavage (Fig. 5).

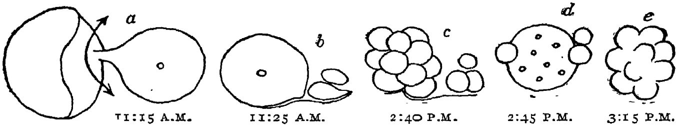

FIG. 5.—(*a*) Endoplasmic exovate being cut off to include part of original cortex. (*b*) The piece inseminated. (*c*), (*d*), and (*e*) Ineffectual attempts at cleavage (Chambers,

It is significant that these fluid spheres from the interior of the mature unfertilized egg withstand disintegration, whether nucleated or not, for a longer period than do fragments containing cortical material.

It follows from these facts that the part of the starfish egg chiefly concerned in development lies in its cortex. The interior when separated from the cortex is incapable of developing. On the other hand, an egg fragment consisting mainly of cortical material is able to carry on its usual life-activities.

The cortex and interior of the starfish egg differ also in their behavior to various cytolytic agents (Page, Chambers, and Clowes, in press). In hypotonic electrolyte solutions, for example, the cortex cytolyzes more readily than does an isolated portion of the interior surrounded by its surface film. On the other hand, the reverse is the case when digitonin is used as the cytolytic agent.

3. *The protoplasmic surface film:*

For a study of the surface film of protoplasm, the unfertilized starfish egg is an admirable object.

The eggs are removed from the ovary and suspended in a hanging drop over a Barber moist chamber. With micro-dissection needles the pellicle of the egg with its investing coat of jelly is then removed. Such an egg still possesses a more or less solid cortex. By tearing through the cortex the interior can be made to flow out into the sea water where it quickly becomes invested in a film which is apparently as fluid as the underlying cytoplasm. This surface layer has no apparent thickness even when viewed with the dark-field illumination. Probably Gaidukov (1910), in his dark-field investigations, included the cortical layer of protoplasm when he described the film as having an appreciable thickness.

That the surface on the exuded cytoplasm is fluid can be shown in the following way: If the egg be gently compressed, the part of the egg covered by the new surface bulges and cytoplasmic granules are carried up through the center of the bulge and those which reach the surface can be actually seen flowing backward in the surface film. This back flow in the surface film is best seen with the dark-field illumination.

Although the surface film is quite resistant to gentle manipulation, it is nevertheless in a very delicately balanced state of equilibrium, and is not at all a boundary between two immiscible fluids (cf. Clowes, 1916). It can be easily destroyed by snapping the tip of a micro-needle across it, whereupon a wave of disintegration travels rapidly over the whole film. The cytoplasmic contents then merge in the sea water, where the granules scatter and disappear (cf. p. 245).

A film which forms on the torn surface of an egg is continuous with that portion of the egg which still possesses the intact original cortex. If this new surface film be torn so that it disrupts, the disruption will tend to spread only to where the original, more solid cortex is still intact. This indicates that the cortex serves as a mechanical support in maintaining the integrity of its surface film possibly analogous to the way in which a porous filter holds up a soap film.

The surface film of blood cells and of tissue cells isolated in blood plasma or serum is extremely susceptible to mechanical injury unless special precautions are taken to maintain conditions as normal as possible. The human red blood corpuscle furnishes an interesting example of a rapid breakdown of the surface film. When the corpuscle is punctured with a very fine glass micro-needle the hemoglobin immediately diffuses out all over the cell and leaves behind a transparent glutinous mass which can be torn into strands (Chambers, 1915*b*).

Another good example is the ciliated cell obtained from the ovary of the sea urchin. These cells, which are about 15 micra in diameter, can be easily isolated with the micro-needle, but when once isolated it is quite a chase to secure one. An active cell can be impaled on the end of a needle without interfering with the movements of the cilia. If, however, the surface of the cell at its base be pricked and ever so slightly torn, a breakdown of the film travels rapidly over the cell (Fig. 6). As the injury reaches the ciliated border the peripheral cilia stop beating and the further advance of this injury can be followed by the successive cessation of more and more cilia until all ciliary action has ceased. The cytoplasm then becomes dissipated in the sea water and the cilia fall apart as motionless filaments.

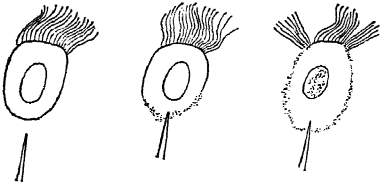

Fig. 6.—Ciliated cell from sea-urchin ovary showing effect of injury with a needle.

4. *Conditions of the protoplasmic surface film formation:*

The readiness of protoplasm to form new surface films varies greatly under different conditions as outlined below.

a) First, we may consider a time factor. If the tearing be done gradually a portion of a cell can be drawn out into a strand which may be torn off without injury to the cell. This phenomenon often occurs spontaneously in amoeboid cells, a part of whose surface happens to stick to a substratum. The adherent part is left behind as the strand which stretches between it and the moving cell is finally broken through (Lewis, 1922). The part of the broken strand connected with the cortex is slowly withdrawn until it finally disappears in the general contour of the cell.

If, on the other hand, the surface of the egg be torn suddenly (Fig. 7*a*) a more or less extensive breakdown of the surface occurs and the exposed cytoplasm immediately disintegrates. Of the visible granules, the first to be destroyed are the macrosomes, which swell and burst. The fluid contents then merge with the hyaloplasm whose microsomes now exhibit active Brownian movement. While this is happening, surface films appear which sweep around masses of the disorganized area, converting them into spherules of all sizes con-

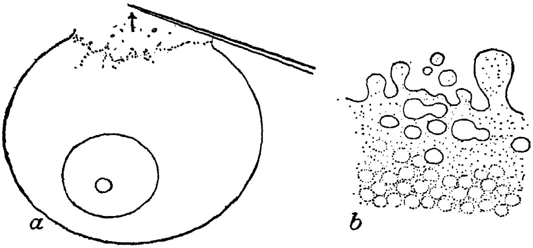

Fig. 7.—(*a*) Disintegrative effect of a shooting tear through cortex of starfish egg. (*b*) Magnified sketch of the disintegrative process.

taining very fine particles in active Brownian movement (Fig. 7*b*). These spherules swell and ultimately burst until the entire mass become completely dissipated. Frequently, the hyaloplasm coagulates into a stringy network whose meshes are occupied by the continually swelling and bursting macrosomes. In all this commotion the observer is struck with a succession of attempts at the formation of surface films which are as continually being broken down. Occasionally a film will sweep around a part of the cytoplasm which is not disorganized, converting it into a globule of apparently healthy cytoplasm. Such a globule maintains its normal appearance so long as it is surrounded by its protective film. It is only the globules of disorganizing material which swell and burst.

The hyaloplasm also tends to form films at the boundary between the disorganizing and healthy cytoplasm. In this region a surface film will appear within what appears to the eye to be healthy hyaloplasm, but which is rapidly being permeated by the sea water. If this film can quickly reach the intact film which still surrounds the rest of the cytoplasm, then all further disintegration ceases and the cell remnant gradually rounds up. That this remnant consists of living protoplasm is demonstrated by the fact that on being fertilized it will segment and develop into an embryo. On the other hand, if the sea water affects the cytoplasm beyond the edge of the advancing film, then disorganization takes place behind it, and the film gradually disappears. Another film then forms ahead of the advancing wave of destruction. In this way several successive films may form and break down before the destruction is finally stopped by a film which persists.

b) Second, the formation of the surface film depends largely on the environing medium. This is seen when a tear is made at one spot on a starfish egg immersed in a hypotonic solution of sea water (70 parts sea water plus 30 parts fresh water). The breakdown of the film travels in a wave which sweeps around the egg from the torn spot while the underlying cytoplasm undergoes complete disintegration (Page, Chambers, and Clowes, in press).

c) Third, the ease with which a film forms on the cytoplasm of a starfish egg also depends to a certain degree on the physical state of the cytoplasm. On tearing cytoplasm which has been rendered very fluid by immersing in alkaline sea water, it is almost impossible to produce disintegration. A protective film rapidly forms on the cut surface. Such an egg can be cut into any number of pieces which immediately round up. When cytolysis once begins, it spreads rapidly through the cell.

If the cytoplasm is rendered more solid by immersing in acid sea water, the surface film does not form so rapidly, so that when the egg is torn extensive cytolysis easily occurs.[1]

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[1] These specific reactions to acid and alkali occur only when the eggs are torn while in the solution. If they are first returned to sea water and then operated upon they behave like normal eggs no matter whether they had been treated with either acid or alkali, except for the

d) Fourth, the readiness to form surface films varies in the protoplasm of different cells. The starfish egg, for example, possesses an extraordinary capacity to form films over torn surfaces whereas the sea-urchin egg does not. Page and Clowes (1922) showed that the behavior of the starfish and sea-urchin eggs to the cytolytic agents, saponin and hypotonic solutions, indicates a fundamental difference in the nature of the protoplasm of the two species. They found that the starfish egg exhibits an extraordinary resistance whereas the sea-urchin egg is very susceptible to saponin. The reverse is true in their behavior to hypotonic sea water. Page (1923) isolated an isomer of cholesterol from the starfish egg, which he termed asteriasterol, and has confirmed Mathews' findings regarding the presence of true cholesterol in the sea-urchin egg. Page has suggested that the difference in behavior of the two species to the cytolytic agents mentioned may be due to this fundamental variation in their sterol content.

e) Fifth and last, the stability of the surface film varies in different species of eggs. When once formed it is often difficult and in many cases impossible to cause contiguous surfaces to fuse and to disappear. This capacity varies in different species. In echinoderm eggs a complete fusion seems to be almost impossible, not only between separate eggs (cf. Goldfarb, 1917), but also between freshly produced fragments. On the other hand, annelid and nematode eggs, which are much more fluid than echinoderm eggs, can be easily made to fuse. The blastomeres of *Chaetopterus* (an annelid), for example, are very fluid, especially when compressed in a shallow hanging drop. By slightly tearing across the surface of two contiguous blastomeres with a needle, fusion readily takes place. This lack in stability of the surfaces on *Chaetopterus* blastomeres is also suggested by the experimental production of multinucleated unsegmented larvae (F. R. Lillie, 1906).

5. *The semi-permeability of the protoplasmic surface film:*

We have so far discussed the purely protective nature of the surface film of living protoplasm. In addition to this, micro-injection experiments (Chambers, 1922*b*) have shown that this film is also responsible for maintaining the semi-permeable properties of protoplasm (cf. p. 152).

These experiments consisted in the injection of NH_4Cl and $NaHCO_3$ into starfish eggs stained with neutral red. In the case where NH_4Cl was used, the injected area immediately changed to a red color and then underwent

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following important point: Starfish eggs treated with acids have their investing egg membranes toughened. With alkali treatment, the membrane is very much softened and may become negligible. On being returned to sea water the condition of the egg membrane remains unaltered although the consistency of the cytoplasm of the egg changes.

Smith and Clowes (1924) found that eggs, when shaken, break into fragments more readily after alkali treatment. This can be explained at least in part by the absence of a sufficiently tough investing membrane.

cytolysis. The color change and accompanying cytolysis spread from this area till it reached the cortex of the egg which disintegrated from within outward. In some cases this spread was arrested by the formation of a surface film which converted the injected and disintegrated area into a vacuole.[1] This experiment demonstrates that $\frac{1}{2}$M NH_4Cl, which causes an alkaline color change within eggs when the eggs are simply immersed in the solution so that its effect is transmitted only through the surface film, will, when injected into the interior of the eggs, produce the acid color change and accompanying cytolysis characteristic of free HCl.

When $NaHCO_3$ was introduced into a stained egg the injected area immediately turned yellow and cytolysis with liquefaction took place. The change to a yellow color and accompanying cytolysis spread throughout the cell. This shows that NaOH, which does not appreciably penetrate the surface film from without, will exert its characteristic effects if introduced directly into the interior of the cell.

The semi-permeability of a living cell is a function of its surface film. It is immaterial whether this film be that of the original cortex, a film newly formed over a cut surface, or a film that surrounds an artificially induced vacuole within the cell. As long as a surface film exists, neither the acid of the NH_4Cl nor the alkali of the $NaHCO_3$ penetrate little if at all. On the other hand, if injected beneath the surface film, they freely permeate the protoplasm.

The micro-injection method fully confirms Pfeffer's results which show that substances which are readily permeable when introduced directly into the interior of the cell cannot enter the cell when the surface film intervenes. Pfeffer's experiment was apparently not as drastic as would appear to be the case, for hydrochloric acid in proper dilution penetrates to a negligible extent only (Lillie, Clowes, and Chambers, 1919; Chambers, 1922*b*), and, if anything, tends to set the protoplasm into a reversible jelly (Chambers, 1920). In Pfeffer's experiment it apparently fixed the cortex of the plant-cell protoplasm so that it supported the surface film during the time required for the experiment.

6. *Conclusions regarding the physical nature of the protoplasmic surface film:*

The film which forms on the surface of protoplasm is an organized structure and not analogous to a simple interfacial film. The facts that a time factor is involved in its production, that it protects the underlying cytoplasm from disintegration, that its durability depends not only upon the environing medium but also upon the specific constitution of the protoplasm from which it forms, and, finally, its semi-permeable properties, all point to its being a definite organized structure.

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[1] The surface films around normally existing or artificially induced vacuoles within the protoplasm of a cell are analogous to the film on the exterior of the cell. Taylor (1923) regards the wall of the contractile vacuole in ciliates as being in the gel state. If true it must be very easily reversible because the vacuole can be cut up with impunity and each piece at once rounds up into a perfect sphere. It is, however, possible to burst the vacuole within the amoeba and to cause its contents to disappear. This can be done by compressing it with the tip of a blunt micro-needle.

The protoplasmic film is in all probability a combination of both passive and chemicophysically active parts. The passive part may be composed of certain cell constituents both organized and unorganized which collect on the surface and which may give to it an appreciable thickness. We must distinguish from this an active film whose instability is such that it can be maintained only in the presence of the passive part.

IV. THE DIFFUSION OF INJECTED SUBSTANCES THROUGH THE CYTOPLASM

Probably the first to inject substances into the living cell was Barber (1911*b*). Barber injected yeast cells, bacteria, and various solutions into the cavity of the comparatively large cells of the plant *Nitella*. He found that the yeast cells and bacteria grow and multiply in the cell vacuole without injuring the protoplasm until they increased in such numbers as to rupture the cell. Some of the solutions, e.g., osmic acid, $HgCl_2$, KOH, chloroform, and ether produce local injury which may or may not be followed by a general death of the cell. NaCl, methylene blue, and water produced no effect. Kite (1913, 1915), using Barber's method, but mainly by blowing into his pipette through a rubber tube, attempted to inject a large number of basic and acid dyes, crystalloids, and water directly into the protoplasm of various plant and animal cells. He secured varying results owing to his inability to appreciate disintegration effects and the readiness of protoplasm to form surface films over torn surfaces (cf. p. 258). Most of his results can be explained by the fact that even when the pipette does penetrate the surface of the protoplasm a new surface film may easily form and become continuous through the tear with the original surface of the cell. The mercury pipette method of Barber (1914) involves extremely difficult technique, but has been successfully used for injecting water, hydrochloric acid and mustard gas into the starfish egg (Lillie, Clowes, and Chambers, 1919). A much more efficient and comparatively easy method has been recently described (Chambers, 1922*a*). With this method a number of substances have been injected into the cytoplasm of the amoeba and marine eggs.

1. *Injections into the amoeba:*

The results of these injections have been published in part (Chambers, 1920). All the aqueous solutions injected (water, aqueous solutions of various dyes) produce a swelling of the cytoplasm and a dilution effect on the granular contents. No vacuoles were produced.

The injection of water, distilled or tap water, in amounts approximating the volume of the nucleus, causes a temporary cessation of amoeboid movement. The cytoplasmic granules are moved from the spot of the injection but slowly return again. Within a short space of time the amoeba returns to its normal condition. If the injection be greater in amount, the body of the amoeba swells up like a balloon. After a few seconds the endoplasm with the granular ectoplasm contracts into a ball surrounded by a dilated hyaline zone. This phenomenon gives one the impression that the granular mass of the amoeba, by contracting, had squeezed out the contained water which then collected between it and the external pellicle. If the injection be directed into the center of the amoeba the dilated hyaline zone completely surrounds the contracted granular material. If, however, it be out of center, the squeezing together of the endoplasm and of the granular ectoplasm produces a localized blister (Fig. 8*b*).

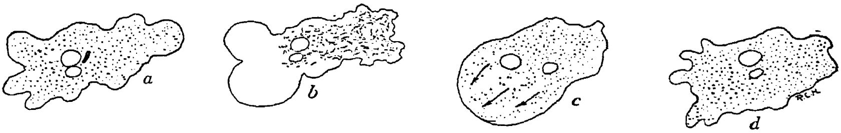

FIG. 8.—Water injected into an amoeba (*a*); immediately reacts (*b*). A few seconds later endoplasm flows (*c*); with complete recovery (*d*).

An amoeba so treated can recover entirely in one of two ways. If the injected water be not excessive the endoplasm after several seconds gradually expands and flows again (Fig. 8*c*), and the external pellicle loses its distended state as if the water were slowly exuding through it. Within a few minutes the amoeba is again back to its former state (Fig. 8*d*). If, on the other hand, an excessive amount of water has been injected, the water collects on one side into a large blister which rounds up and eventually is pinched off from the main body of the amoeba which then reassumes its normal activities.

The injection of "basic" dyes (Janus green, pyronin, methylene blue, neutral red) produces a localized coagulation of the cytoplasm and stains the coagulated area only. Figure 9 shows the effect of neutral red which is the least toxic of these dyes. The coagulated area produced by the injection stained a bright rose red. The streaming movements in the amoeba continued in the direction of the arrow, while the coagulated area was left behind. Gradually various granules in the cytoplasm took up the dye and the coagulated area slowly became dissipated until after several minutes the entire body of the amoeba was filled with scattered red granules.

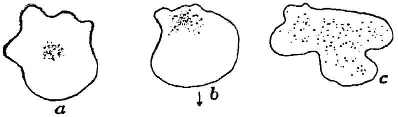

FIG. 9.—Neutral red injected into an amoeba

The injection of "acid" dyes in aqueous solution (trypan red, trypan blue and acid fuchsin, eosin, alizarin) produces an opposite effect. No coagulation occurs, and the dye rapidly diffuses through the cytoplasm and liquefies it. When death ensues the liquefied state is maintained. The dye then diffuses out of the amoeba. If the amount of injection be minute the area immediately about the injection liquefies. The rest of the cytoplasm contracts as the liquefied colored area collects under the pellicle, which swells into a rounded blister. The blister is finally pinched off while the rest of the amoeba recovers.

Various oils were also injected. A drop of chloroform or xylene about the volume of the nucleus forms a spherule and the amoeba rounds up and quickly coagulates. Paraffin oil and olive oil produce no effect, the droplets injected being carried about with the streaming of the cytoplasm. If two droplets of oil be injected they run into one drop on touching and give no indication of a differentiated vacuolar wall of cytoplasm having been formed about them.

2. *Injections into echinoderm eggs:*

Olive oil, paraffin oil, sperm oil, starfish oil (kindly furnished me by Mr. Page, see his article, 1923) persist as droplets when injected.

The injection of various electrolyte solutions have been attempted in order to ascertain the action of various electrolytes on the formation of the protoplasmic surface film. These experiments are still under way. The difficulty of the procedure lies in the ease with which disintegration of the cytoplasm occurs upon injection. About the disintegrated area a protoplasmic surface film forms so as to convert the area into a vacuole of disintegrated material (cf. p. 260.)

A minute quantity (1–2 cu. micra) of $\frac{1}{2}$M NaCl can be injected into the sand-dollar egg without causing the formation of a vacuole or bringing about any apparent disintegration of the cytoplasm. The reaction to this injection was a flow of cytoplasmic granules away from the tip of the micro-pipette so as to leave a hyaline area of about 8 microns in diameter (Fig. 10). Within ten to fifteen seconds the granules again filled up the area and the egg resumed its normal appearance.

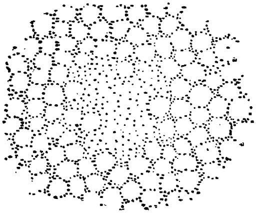

FIG. 10.—Area in cytoplasm of starfish egg injected with $\frac{1}{2}$M NaCl.

3. *Results from the injection experiments:*

1. Aqueous solutions can be injected into the protoplasm of the amoeba and the echinoderm egg without producing an interfacial film between the injected liquid and the protoplasm.
2. The oils injected (olive oil, paraffin oil, sperm oil, starfish oil, chloroform, xylene) immediately round up into drops.
3. Chlorides of basic dyes coagulate whereas the salts of acid dyes liquefy protoplasm, an effect probably attributable to the free acid of the "basic" dyes and to the free base of the "acid" dyes.
4. Dyes which do not penetrate the living amoeba from outside (e.g., eosin) rapidly diffuse through the cytoplasm when injected.
5. The injection of aqueous solutions into the cytoplasm of echinoderm eggs is apt to cause a disintegration of the cytoplasm whereupon a protoplasmic film forms and converts the disintegrated area into a vacuole.

From these results we may conclude that the internal protoplasm is freely permeable to water whereas it is not to the oils which have been injected. It is also shown that the protoplasmic surface film possesses properties of impermeability which the protoplasm within the film does not possess.

V. THE CELL NUCLEUS

1. *The physical state of the interkinetic nucleus:*

It has always been a question whether structural differentiations exist within the living nucleus during its interkinetic or so-called resting period. During mitosis, on the other hand, distinct structures, the chromosomes, are plainly visible. Indeed, the discovery of chromosomes was made by Schneider in 1873 from observations on living material.

When the micro-dissection method was first applied to the study of the nucleus, Kite (1913*b*) came to the conclusion that the typical cell nucleus is a "gel" containing more concentrated areas in the form of granules, and an imperfect network. An exception to this is the nucleus of the immature starfish egg which he found to be a liquid body inclosed within a membrane.

The difficulty of interpreting the physical state of the nucleus is due to the extraordinary ease with which its substance sets into a relatively firm jelly. This is in accordance with the fact that one of the chemical constituents of the nucleus is nucleic acid, and this has remarkable powers of forming gels (Mathews, 1920).

With improved methods, it has been definitely shown, however, that the interkinetic nucleus in every living metazoan cell so far studied is fluid and possesses no visible structure except for one or several nucleoli and a delicate investing membrane (Chambers [germ cells], 1914, 1915*a;* [ova], 1915*b*, 1917*a*, 1918*b;* Chambers and Schmitt [pancreas, kidney, epithelium, muscle, nerve], in press). If the needle be carefully inserted into the nucleus no coagulation takes place and the needle tip can be moved inside the nucleus from one side to the other without meeting any appreciable obstacle. If a nucleolus is present it can be pushed out of its normal position and will not move back when released as would be expected if a tenuous elastic network were interspersed throughout the nucleus.[1]

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[1] In a hyacinth and onion root tip it has so far been impossible to ascertain whether the nucleus normally is solid or liquid. It can be pushed about in the cell without being deformed. If it is liquid it must be in a state of considerable turgor. On being punctured or being sucked into a micro-pipette, it behaves like a mass of highly viscid jelly. With the needle, strands can be pulled out which on being released tend to curl up. In Protozoa both the micro- and macronuclei appear to be distinctly gelatinous bodies. They adhere tenaciously to the needle and can be pulled out of the cell and torn into viscid gelatinous strands (Taylor, 1920). Peebles (1912), by cutting experiments, noted variations in the consistency of the macronucleus. During the vegetative period of the *Paramoecium* she described it as fluid with a hard shell which cracks on crushing. At other times she found that it could be cut like cheese.

The method of dark-field illumination also reveals no internal structure in the interkinetic nucleus. From the lack of ultramicroscopically visible structures Della Valle (1913) concluded that the nuclear material is not colloidal in nature. Rhumbler (1914), however, criticized this statement by pointing out the fact that colloidal emulsions need not exhibit their heterogeneous structure. An emulsion of oil and gum arabic which distinctly exhibits an alveolar structure in ordinary light appears perfectly homogeneous and optically empty under the ultramicroscope.

The nuclear membrane is a morphological membrane, and can be thrown into wrinkles when the nucleus is pressed out of shape. This can easily be done in the relatively large germinal vesicle of immature ova, and was so recorded by Albrecht (1898) who made his observations by crushing the egg cells between cover glass and slide.

By tearing the cytoplasm of a cell, the nucleus may be removed intact. It then either swells until it bursts, leaving nothing behind except sometimes a small, coagulated remnant (Chambers, 1921*e*), or it may set into a coagulated body which can be cut into pieces. The swelling and subsequent dissipation of liquid contents is especially true for the large germinal vesicles of eggs. The same phenomenon occurs with the nuclei of somatic cells dissected in lymph or Ringer's fluid. Frequently, however, the nucleus sets into an irreversible jelly as soon as it is removed from the cell. This explains the frequent presence of large numbers of naked nuclei in preparations of fresh smears of cellular tissue. As long as the nuclear membrane is not torn the nucleus can be moved about, and may be considerably indented without injurious effects.

In the immature starfish egg it has been possible, by compressing the nucleus to cause nuclear material to protrude through a rupture in its membrane. The protrusion is immediately bounded against the cytoplasm by a delicate film. On being released from compression the nucleus tends to revert to its original spherical shape (Chambers, 1921*e*).

Usually, however, when the nuclear membrane is torn, a very striking phenomenon occurs. After several moments' pause the cytoplasm immediately surrounding the nucleus disintegrates and liquefies (Fig. 11*a*, *b*, and *c*). If the rupture of the nucleus be roughly done, the disintegration of the cytoplasm spreads rapidly until the entire egg is involved. If the nuclear membrane be gently torn, the disintegrative process is slow and is quickly limited by a protoplasmic film (cf. p. 258) which forms at the boundary between the disintegrating and the healthy cytoplasm (Fig. 11*d* and *e*). Sometimes the film breaks and the disintegration spreads, to be stopped again by a second film. It is significant that the injurious effect on the cytoplasm apparently spreads at about the same time from the entire surface of the injured nucleus. This is analogous to the exudation of hemoglobin from injured red blood corpuscles previously mentioned.

Within the nucleus itself the immediate effect of the injury is a dissolution of the nucleolus. A nuclear remnant tends to persist after the injury in the form of a hyaline sphere lying within the disintegration products of the cytoplasm. On being touched with the needle it fades from view.

With the micro-pipette it is possible to extract the fluid contents of the nucleus and to inject it into the cytoplasm of another cell with destructive results. This operation must be performed rapidly because the nuclear substance becomes innocuous if it remains more than five or ten seconds in the micro-pipette.

Although the nucleus on injury either goes into solution or sets into a rigid homogeneous gel, it may give evidence of a network resembling the chromatin network familiar in fixed material. This I have occasionally observed in the germinal vesicle immediately on being removed intact from the egg. Hazy granular filaments gradually appear immediately under the nuclear membrane. They, however, soon disappear as the nucleus swells and finally goes into solution in the sea water.

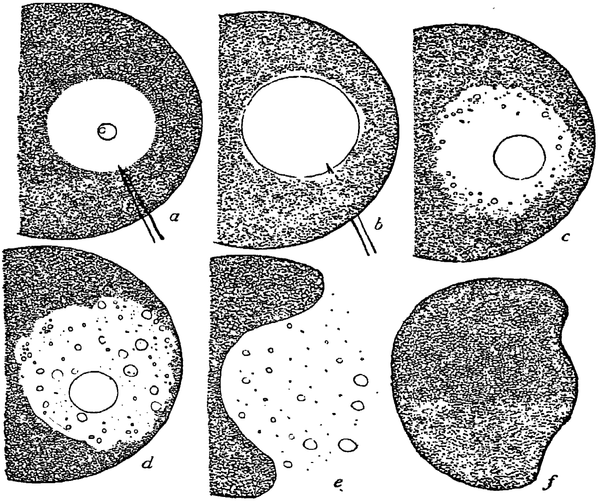

FIG. 11.—Disintegration of cytoplasm of a starfish egg by puncturing the nucleus

In somatic cells the appearance of a granular network occurs more frequently. Sometimes, merely rough handling will cause a heavy granular network to appear in the nuclei. Coagulating reagents, such as acetic acid fumes or ether vapor, produce the network instantly.[1] On the other hand, formalin occasionally fixes the interkinetic nuclei as homogeneous bodies with no evidence of a network structure.

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[1] A frequent feature of a coagulated nucleus which contains one or more nucleoli is the way in which anastomosing granular strands radiate more or less irregularly from the nucleoli. This is what one would expect to occur when coagulation strands are formed about a preformed body.

From what has been observed in living cells and from the results of microdissection studies, it would appear that this latter reagent presents a more faithful picture of the living state.

2. *The chromatin filaments in the male germ cell of the grasshopper (Dissosteira Carolina):*

The structure which appears in the injured nucleus of the growing spermatocyte is quite different from that which has just been described as occurring in the injured interkinetic nucleus. Instead of forming an anastomosing network, the strands which appear develop into more or less separate granular filaments. This fact is of considerable importance because of its bearing on the appearance of the chromosomes characteristic of the dividing nucleus. The spermatocyte is from its very early stages a typical prophase nucleus, i.e., changes are taking place within it preparatory to the appearance of chromosomes.

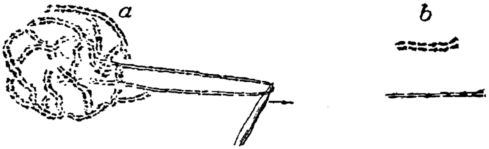

FIG. 12.—(*a*) Loop of chromatin filaments of spermatocyte nucleus stretched with needle. (*b*) Portion of filament stretched and unstretched.

The nucleus of the growing spermatocyte is an optically homogeneous sphere containing two or three hyaline bodies, the nucleoli. Under dark-field illumination it is optically empty except for the faintly illuminated nucleoli and for a delicate bright line around its border. One of the nucleoli is elongated and generally lies immediately beneath the nuclear membrane. The others are globular.

On pricking the surface of the nucleus, hazy streaks of granules appear in its hyaline matrix. The streaks steadily increase until the nucleus becomes filled with a tangle of granular filaments. If the nuclear membrane be now torn, the membrane disappears, but the filaments persist and gradually shrink and clump together into an amorphous glutinous mass. Before this occurs a loop from the tangle can be pulled out into an attenuated strand (Fig. 12*a*). On being released the loop slowly retracts and thickens again. Figure 12*b* shows a portion of the filament in the stretched and in the unstretched condition. The illustration shows clearly that the filament consists of an extensible hyaline core closely invested by clumps of granules. On stretching the core, the investing clumps are drawn apart. When the filament is released, the clumps return to their original positions. This granular filamentous structure in the nucleus can be entirely destroyed by sucking the nucleus into a capillary pipette, the bore of which is smaller than the diameter of the nucleus. On ejection from the pipette, the nuclear material comes out as a homogenous, glutinous mass with no structural elements whatever.

Figure 13*a–d* shows the successive changes which occur in a spermatocyte nucleus during the twenty-five minutes after the cell had been injured by being punctured with a needle. The spherical nucleoli usually, but not always, fade from view; the elongated peripheral nucleolus, on the other hand, steadily becomes more and more distinct. Within one minute after the puncture, the hitherto optically structureless nucleus begins to give evidence of hazy granular streaks which give the impression of being produced by a linear condensation or precipitation of granules in the hyaline nuclear substance (Fig. 13*b*). Whether this results in the formation of a number of separate or only of a single coiled filament, it is impossible to judge. As the granules increase and coarsen, the filamentous structure slowly thickens until one begins to appreciate that the body of the filament consists of a hyaline core with granules adhering to its surface (Fig. 13*c*). The elongated nucleolus has become distinctly stouter. During this time the injured nucleus swells and then shrinks in size.

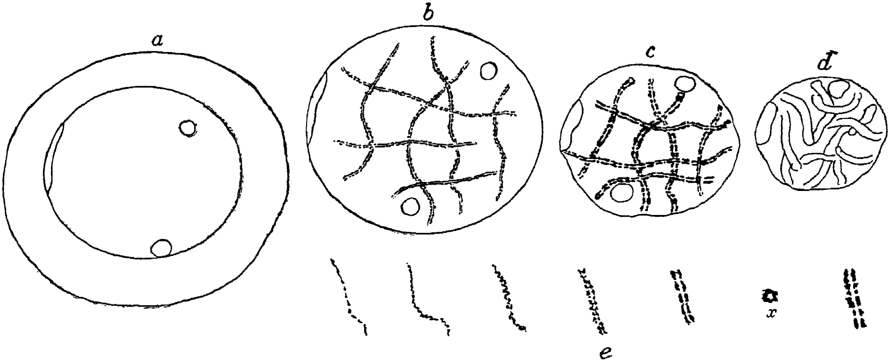

FIG. 13.—(*a*) Living grasshopper spermatocyte (mitochondria not shown). (*b*), (*c*), and (*d*) Successive stages in nucleus upon injury. (*e*) Detail to show gradual thickening of nuclear filaments. (*x*) End view of a filament.

Within ten minutes after the puncture, the filaments are distinctly thickened and appear lumpy because of the irregular grouping of the investing granules. In side view the filaments appear double. In optical section, however (Fig. 13*c* and *x*), they are cylindrical with the groups of granules arranged about a hyaline core. The elongated nucleolus has still further thickened and shortened while the globular nucleoli fade from view. Successive stages in the thickening of a portion of one of the strands are shown in Figure 13*e*.

During the next ten minutes, the granular clumps fuse together as the filamentous loops further shorten and thicken until they are transformed into a number of homogeneous, rodlike bodies (Fig. 13*d*). The elongated nucleolus has also shrunk into a definite body, which now resembles the other bodies lying within the nucleus. They are precociously formed chromosome-like bodies, one of which has been traced directly from one of the nucleoli, while the others have arisen by a series of transitions from what appear to be linear condensations of granules out of the hyaline nuclear substance.

Not all spermatocytes behave in the manner just described. Frequently, one comes across a cell the mechanical stimulation of which, instead of producing simple granular filaments, causes the appearance in the nucleus of ill-defined granular condensations which rapidly resolve themselves into the early prophase chromosomes, with the shapes of crosses, rings, and double V's, familiar to cytologists (Fig. 14*a*). The irregularly disposed clumps of granules give a ragged outline to these long and slender structures. Gradually, as one watches them, they shorten and their granular clumps coalesce until they are transformed into compact and homogeneous bodies without losing their characteristic shapes (Fig. 14*b*).

This artificially induced appearance of the chromosomes is unaccompanied by the dissolution of the nuclear membrane. The chromosomes if left alone gradually clump together and finally become indistinguishable in an irregular, glutinous mass. They are very viscous and adhere to the needle. If one of the early prophase chromosomes with ragged granular outlines be seized with a needle and rapidly pulled across the field so as to stretch it, the granules disappear and the whole substance becomes homogeneous. The entire nuclear substance is somewhat viscid, and the chromosomes cannot be taken out of the nucleus entirely free of the medium in which they lie. If the nucleus be taken out of the cell in Ringer's fluid, the nuclear matrix absorbs water, swells, and gradually disappears. The chromosomes are thus set free, and they then also slowly swell and go into solution.

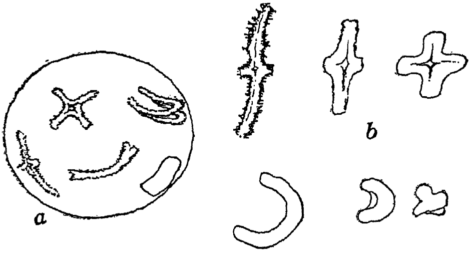

FIG. 14.—(*a*) Prophase chromosomes brought into view by pricking the cell. (*b*) Successive changes in isolated prophase chromosomes.

Occasionally, one may find spermatocytes with late prophase chromosomes which are already visible as hazy bodies within the nucleus, although the cells containing them have not been punctured or otherwise injured. On puncturing such cells, the outlines of the chromosomes become rapidly more and more distinct. They then shorten, and their granular aspect disappears until they change into chromosomes exactly resembling metaphase chromosomes except that they lie within an intact nuclear membrane.

These different ways in which the prophase nuclei react to mechanical injury enable one to distinguish three stages in the development of the growing spermatocyte: (1) Nuclei of the first stage respond to the injury by revealing delicate granular filaments which shorten and thicken into homogeneous-appearing rodlets. (2) In the second stage, injury causes the appearance at once of early prophase chromosomes which precociously resolve themselves into typical metaphase chromosomes. (3) In the third stage, prophase chromosomes are already visible in the uninjured nucleus and injury simply results in accentuating their visibility and in accelerating their transformation into compact, metaphase chromosomes. We, therefore, see that injury to the nucleus initiates a series of transformations, and it is significant that, with each successive stage, the transformations start at a point which is ahead of that of the preceding stage.

The fact that well-differentiated structures are spontaneously beginning to be visible in nuclei of the third stage, and the fact that these structures stand out with greater clearness on injury, suggest the possibility that the granular chromatin filaments are already present as such in the earlier stages and are not formed *de novo* as an effect of injury. Their invisibility may be accounted for by the identity of their refractive index with that of the nuclear substance in which they lie.

We may, therefore, infer that mechanical injury to a prophase nucleus brings at once into view the structures which have been slowly developing up to the time that the nucleus was injured, and then accelerates their further changes into precociously formed chromosomes.

Evidence has already been presented regarding the absence of structure in the interkinetic nucleus. The only structure which can be produced by injury in such a nucleus is a network coagulum. It is quite otherwise with a prophase nucleus. Here we have a nucleus preparing for mitosis at which time distinctly structured chromosomes spontaneously appear. Evidently, injury to such a nucleus simply accelerates in a more or less normal manner the regular process in the formation of the chromosomes.[1]

3. *The mitotic spindle:*

In the late prophase stages of the spermatocyte the chromosomes are plainly visible, and are distributed mainly in the periphery of the fluid nucleus. On tearing the nuclear membrane, the nuclear matrix goes into solution and the chromosomes fall out.

During the metaphase stage the nucleus loses its spherical shape and becomes spindle-shaped or fusiform. The sharply defined nuclear membrane fades from view and the chromosomes collect in the equator of the spindle. This constitutes the mitotic spindle. Division takes place by a separation of the chromosomes at the equator and their migration to the two poles of the spindle where they reconstitute the daughter-nuclei.

In coagulated material the spindle is filled with fibers which extend from the poles to the equator where many of them are attached to the chromosomes. In the living cell the spindle is a hyaline body with no visible structure except for the chromosomes at its equator. It possesses a certain amount of density, for it can be moved bodily through the cytoplasm of the cell as has already been shown by Morgan (1910), Spooner (1911), Conklin (1917), and Chambers (1917*b*), in marine ova, and by Němec (1915) and Andrews (1915) in plant cells.

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[1] Pentimalli (1912) found a pronounced difference between the contents of an interkinetic nucleus and the prophase and metaphase chromosomes in their behavior to the passage of an electric current.

The spindle very readily sets into an irreversible jelly upon mechanical injury and in this way resembles the nuclear substance and differs from the cytoplasm.[1]

In the sand-dollar egg, the needle has demonstrated that the spindle changes in viscosity during mitosis. When the chromosomes are in the equator, its viscosity is comparatively high. As it lengthens, it still remains viscid, but by the time the chromosomes have reached its poles and the amphiaster (cf. p. 291) is fully developed it becomes distinctly fluid. A needle inserted into it can be moved back and forth without meeting any resistance. On moving the needle out of the spindle area one at once meets with the resistance of the investing granular cytoplasm which, in comparison, is highly viscid. When the needle is removed, the cytoplasm moves back into position again. A slight tearing movement of the needle, however, causes the spindle fluid to flow out and form a bulge on the side of the spindle.

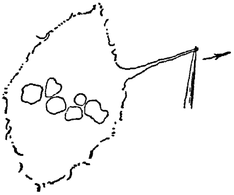

FIG. 15.—Isolated metaphase spindle from which a strand is pulled out by a needle.

In the pollen mother-cell of the *Tradescantia* (Chambers and Sands, 1923), the spindle is distinctly outlined by the surrounding granular cytoplasm. After the stiff, investing cellulose wall is removed, the peripheral zone of granular cytoplasm can be easily stripped off in a sheet from the hyaline spindle, which maintains its shape even when entirely laid bare. It is jelly-like in consistency throughout, and shows no sign of fibrous structure. By inserting needles the material of the spindle can be stretched, and chromosomes which happen to lie between the needles are stretched also. On being released, the stretched area retracts. The chromosomes are considerably more solid, and they can be removed by tearing away the substance of the spindle.[2]

In the insect spermatocyte (Chambers, 1914, 1915*a*, and in press), the cytoplasm and the spindle substance are both very fluid. A needle may be inserted through the cytoplasm into the spindle, and can be moved from side to side without dislocating the chromosomes unless they are touched with the needle. The cytoplasm readily disintegrates by tearing, whereupon the spindle is set free as a fusiform body with mitochondria of the cytoplasm (cf. p. 274) adhering to its surface. Figure 15 shows one such isolated metaphase spindle from which a viscid strand has been pulled out by a needle. After several minutes the substance of the spindle also goes into solution, whereupon the chromosomes lose their orderly arrangement in the equator. Before the spindle has gone into solution one may insert a needle into the equatorial plate and pull out a chromosome. On dragging out a chromosome there is no evidence whatever of spindle fibers. When one chromosome is dislodged the others also leave their places, and, if they touch, they adhere and clump together into an irregular amorphous mass.

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[1] Foot and Strobell (1905) found that the metaphase spindle comes out as a single coherent structure from earthworm eggs which have been crushed.

[2] These operations were done in a drop of equal parts of 10 per cent saccharose and plant sap. In this solution the spindle substance liquefies within 15 to 20 minutes, whereupon the chromosomes can be easily isolated.

4. *The chromosomes:*

The isolated chromosomes of the *Tradescantia* and of the grasshopper are very similar in their consistency and their behavior to the needle, the main difference being that the plant chromosomes are very resistant to injury whereas the animal chromosome soon disintegrates after removal from the cell.

The isolated chromosome is a body which can be stretched with needles and torn into stringy masses (Fig. 16). In lymph fluid or in Ringer's solution it gradually swells and disintegrates. Disintegration occasionally sets in by the formation of irregular protrusions which may lengthen within a few minutes into attenuated filaments. The tips of the filaments become knobbed and pinch off as droplets. The successive stages in the disintegration of two grasshopper chromosomes are shown in Figure 17*a* and *b*. One of these chromosomes (*b*) developed an attentuated filament which oscillated to and fro for some time before undergoing dissolution.[1]

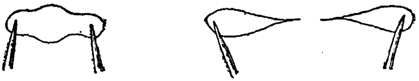

FIG. 16.—Metaphase chromosome being torn by needles.

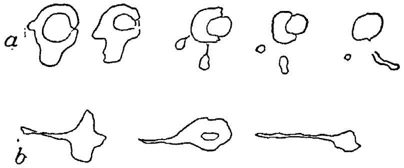

FIG. 17.—Isolated chromosomes disintegrating in Ringer's fluid.

Figure 18 is from three photographs (taken by Dr. Sands) of unstained chromosomes of the *Tradescantia* pollen mother-cells (Chambers and Sands, 1923) isolated and dissected with needles. Figure 18*a* shows two isolated chromosomes. One of them is a definite ring with transverse constrictions.

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[1] This is undoubtedly what Kite and I wrongly interpreted in one of our first papers (Kite and Chambers, 1912) as a spindle fiber. The formation of such filaments and their peculiar oscillatory movements have long been observed in disintegrating protoplasm. Lieberkühn (1870) describes it in red blood cells, and ascribes the movement to the presence of Brownian movement (*Molekularbewegung*) in the immediate environment of the processes. Grasshopper germ cells exhibit this phenomenon very readily when isolated in Ringer's solution, especially if the solution be hypertonic (Chambers, 1914).

The other is almost a closed V. One arm of the V is turned up so as to present an end view. This shows it to be a cylinder possessing a distinct cortex and a central core. Figure 18*b* shows one half of the ring-shaped chromosome, the other half having been dissected away. In Figure 18*c* the chromosome of the previous figure was caught at its two ends by the needles and stretched.

Regarding the consistency and structure of the chromosome we can draw the following conclusions: The metaphase chromosome is a gelatinous and extensible body which may give evidence of a definite structure in possessing a cortex which can be optically differentiated from a central core. This structure is significant in view of the way in which the artificially induced chromatin filaments come to view in the prophase spermatocyte of the grasshopper. Granules appear out of the hyaline nuclear material, and align themselves in rows. As the granules increase and accumulate, their arrangement about a hyaline non-granular core becomes more and more appreciable. The definitive chromosome finally results by a shortening of the core and the fusion of the granules into a hyaline cortex. Sands (1923) has been able by special staining methods to block out definite areas on the surface of the *Tradescantia* chromosome which also indicate its morphologically heterogeneous nature.

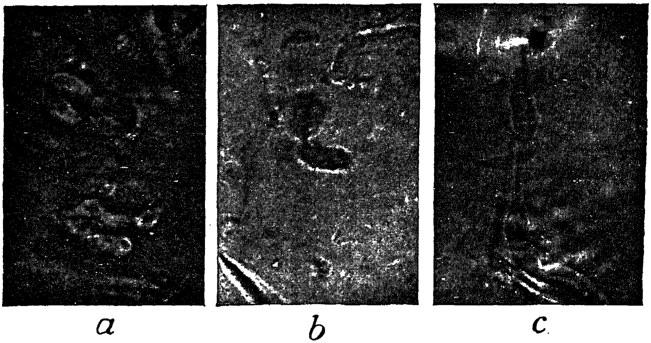

FIG. 18.—*Tradescantia* chromosomes isolated by needles and dissected (Chambers and Sands, 1923).

VI. MITOCHONDRIA IN THE GRASSHOPPER SPERMATOCYTE

The mitochondria are extensively distributed cytoplasmic inclusions, a micro-dissection study of which has so far been made only in the spermatocyte of the grasshopper.

In the fully grown primary spermatocyte, they are minute and highly refractive granules and threadlets, which are uniformly distributed in the hyaline cytoplasm immediately around the nucleus.

During life they appear to be constantly shifting in position and to be disappearing and reappearing in the cytoplasm (Chambers, 1915*a*). By dark-field illumination they are brilliantly illuminated, and are by far the most prominent structures in the cell, the only other structure visible being the spherical nucleus with its pale, silvery outline and faintly illuminated nucleoli. By transmitted light they are visible, although not prominently so.

Injury to a cell by puncturing produces a change in the cytoplasm, which causes the mitochondria to be fixed and to show up more distinctly. On tearing a cell the cytoplasm goes into solution and the mitochondria scatter in the surrounding medium where they exhibit Brownian movement. They persist as such long after the cytoplasm and nucleus have completely disintegrated.[1]

During the metaphase of the dividing cell, the mitochondria closely invest the spindle. When the cytoplasm is disintegrated by being torn, the mitochondria adhere into a network which persists together with the spindle. The substance of the spindle, however, soon goes into solution, leaving the mitochondrial network about an area in which the chromosomes are irregularly dispersed. Spermatozoa, which are swimming about, easily pass through the open meshes of the net. The viscosity of the network material is such that spermatozoa which touch it stick fast, and can release themselves only by violent lashings of their tails. In one case a spermatozoön swam through the mesh into the liquefied spindle area where its tail came into contact with a chromosome. The spermatozöon continued its way with the chromosome adhering to its tail. This peculiar adhesive quality is often one of the first disintegrative signs of protoplasm. The adhesion of the mitochondria into a network about the isolated spindle is a similar phenomenon. As disintegration proceeds the protoplasm goes farther into solution, and certain of its components resolve themselves into non-adhesive degeneration granules which exhibit Brownian movement and quickly disperse.

During the anaphase stage when the chromosomes move to the poles of the spindle, the mitochondria become converted into long strands (Fig. 19*a*). They sweep in broad curves with their ends converging at the two poles of the cell. Frequently the fibrils are massed into several separate groups about the

[1] If the spermatocyte be vitally stained with Janus green ("B" of Hoechst), the mitochondria stain a beautiful blue (Chambers, 1914). The stain, however, apparently fixes the mitochondria. In the presence of Janus green the colored mitochondria persist and steadily increase until they finally coalesce into an irregular clumpy network. Apparently, when once stained, they are unable to pass back into solution and, as fresh mitochondria are constantly forming, the stained mitochondria steadily increase. The end result is the death of the cell upon which the mitochondria lose the stain, which now passes into the nucleus. They likewise lose their color when the cell is torn and the mitochondrial network is set free in the environing medium. The mitochondria can hold the stain only when they are within a healthy, intact cell. On the other hand, the nuclear structures take up the stain only when the cell dies

spindle. When the cell is torn the mitochondrial strands wrinkle and gradually anastomose into an amorphous gelatinous mass (Fig. 19*b*).

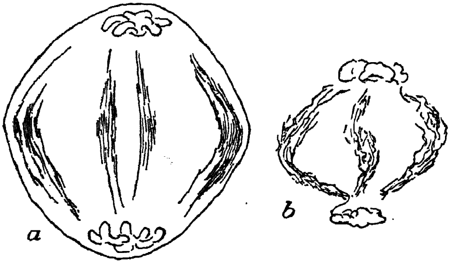

FIG. 19.—Mitochondria in streaks about telophase spindle, *a*, before, and *b*, after destruction of cell.

The spindle and the investing strands maintain a convex barrel-like shape until the cleavage furrow of the dividing cell penetrates for some distance. As the furrow still further deepens the spindle narrows and lengthens. The investing group of mitochondrial strands similarly become narrowed at the equator and gradually take on the shape of an hourglass, with ends projecting into the interior of the daughter-cells.[1]

If one of the two daughter-cells be destroyed while they are still connected, the mitochondrial strands persist as a cluster projecting out of the remaining cell (Fig. 20). The strands quickly wrinkle up along their whole length and gradually shorten. After some minutes the remaining cell may be destroyed by the needle, but the cluster persists for a long time as a wrinkled and tangled mass of filaments (Fig. 20*e*).

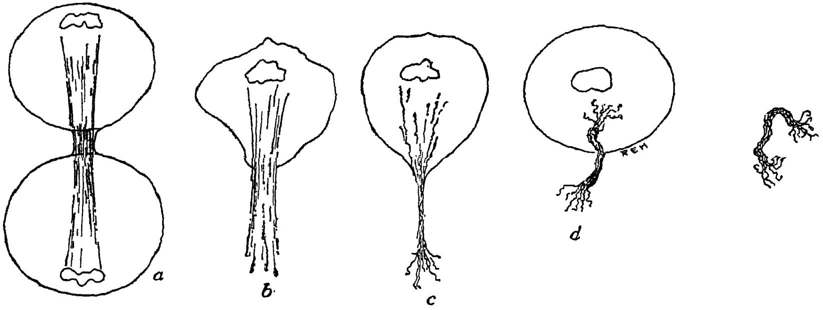

FIG. 20.—Effect on mitochondrial strands of injuring the daughter-cells

VII. VISCOSITY CHANGES IN THE AMOEBA

Probably the first one to suggest that reversible viscosity changes are involved in the formation of pseudopodia was Montgomery (1881). F. E. Schultze (1875) noticed, in *Pelomyxa,* the cessation of the peripheral back flow of granules at some distance from the tip of the pseudopodia, and Berthold (1886) elaborated a theory on the basis of Schultze's observations that the cessation of movement is due to a localized stiffening of the protoplasm. Prior to this the temporary cessation of the flow of granules in the amoeba had been ascribed in a vague way to contraction. More recently Rhumbler (1905, 1910), in connection with Bütschli's (1892) surface-tension theory, pointed out the significance of the reversible colloidal properties of protoplasm to account for the localized growth of pseudopodia and their stiffening.

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[1] In the sand-dollar egg (cf. p. 272) it can be shown that the spindle substance at this stage is more fluid than before. We may, therefore, infer that the narrowing of the spindle is due to a flow of the liquefied spindle material toward the poles where the chromosomes have congregated.

Recently Bayliss (1920), by means of dark-ground illumination, has demonstrated the reversibility of Brownian movement in connection with amoeboid movement. He made his observations on the clear protoplasm in the outer part of the pseudopodia. On applying an electrical stimulus not strong enough to cause sudden retraction, he noted that the continuous shimmering, tremulous movement of the bright points, due to their Brownian movement, ceased almost instantaneously, as if the liquid protoplasm had been frozen. As soon as this happened, he shut off the electric current and, apparently, almost at the same time, the Brownian movement and the flowing pseudopodial extrusion recommenced. Bayliss (personal communication) also detected the spontaneous appearance of Brownian movement in the peripheral cytoplasm of an amoeba at a spot which a moment later bulged to form an actively growing pseudopod.

Jacobs (1922), who found that low concentrations of carbonic acid liquefies protoplasm, succeeded in causing the formation of a protuberance by gently discharging a very small amount of CO_2-saturated water against the body of a quiescent amoeba. In every case a protuberance was immediately extended in the direction of the capillary tube from which the CO_2 exuded. Then, as the CO_2 became more concentrated around the amoeba, all movements ceased temporarily, the amoeba remaining, as it were, congealed in the form in which it happened to be when overtaken by the effects of the dissolved gas. To all appearances the amoeba had undergone a local liquefaction followed by a more general solidification.

It is a question whether we can imply from Jacob's results that pseudopodial formation is necessarily due to external liquefying agents. Edwards (1923), for example, secured similar positive responses with a variety of agents (salt, acids, alkalies) all of which do not necessarily liquefy protoplasm.

With the micro-needle it is very difficult to ascertain changes in the consistency of the amoeba owing to the fact that it tends so rapidly to reverse its state of viscosity by mechanical agitation.

In free living cells other than those of Protozoa and Myxomycetes, L. Loeb (1920, 1921) has shown that amoeboid movement is due to changes in protoplasmic consistency. He found that the formation of pseudopodia in the amoeboid blood cells of *Limulus* is largely influenced by the presence in the surrounding medium of those ions which have a swelling or shrinking effect on gelatin.

Generally speaking, we may conclude that the pseudopodium commences by a liquefaction process. The pseudopodium, however, does not remain liquid (Jennings, 1904). Hyman (1917), in her demonstration of the existence of metabolic gradients in the pseudopod, concludes that liquefaction is the cause of its extension and coagulation or gelation, of its withdrawal and of its active contraction. Dellinger (1906) showed that the amoeba is able to raise itself on the tips of its pseudopodia.

1. *Micro-dissection studies on Amoeba proteus, Leidy:*

The amoeba, when at rest, generally possesses a number of long, tapering pseudopodia, all of which are in a condition of such rigidity that the amoeba may be rolled and pushed about without inducing any change in its shape. If the amoeba be agitated with a needle, centripetal streaming movements start up in the central axes of the pseudopodia, resulting in their retraction. The streaming is at first very slow, commencing at the base of each pseudopodium and extending until the whole pseudopodium is involved. Upon continued agitation the streaming is accelerated until the whole amoeba becomes distinctly fluid with fresh pseudopodia rapidly forming at different spots on its surface. It is significant that the pseudopodia which now form are not only more fluid but are broadly lobate and quite unlike the long, slender, tapering ones originally present. These new pseudopodia cause the amoeba to move about rapidly as if it were fleeing from the needle which is disturbing it. The longer the amoeba be kept in continual agitation, the broader the pseudopodia tend to become. Occasionally (Fig. 21), this results in the conversion of the entire body of the amoeba into a sphere with rolling currents which flow over its surface from one spot and turn in at the opposite pole to flow forward as an axial current. As fast as the axial currents carry materials forward the peripheral currents carry them back so that the amoeba as a whole remains sta-

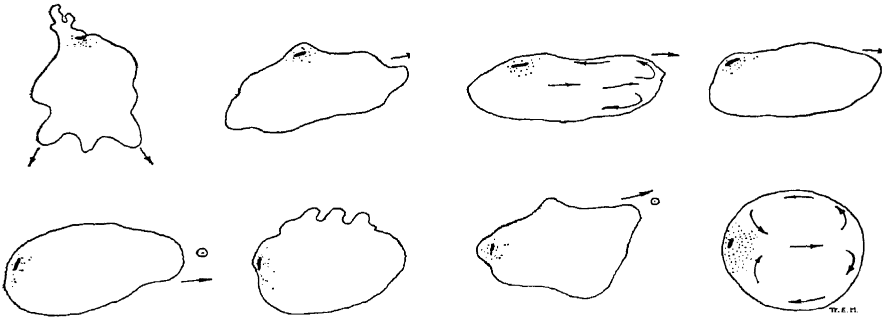

FIG. 21.—Effect on an amoeba of irritating it repeatedly with a needle

tionary. When once an amoeba is brought into this condition I have not observed recovery. The currents may continue for several hours. After the first ten minutes or so they gradually slow down until they finally cease, when the amoeba is found to be converted into a solid spherical coagulum of dead matter.

One of the main arguments raised by Jennings (1904) against the surface-tension hypothesis is the fact already hinted at by Bütschli (1894) that particles of foreign material which accidentally adhere to the outer surface of an amoeba move forward with the amoeba instead of backward as the theory requires. In an amoeba which is moving over a rigid substratum, Jennings found that only the granules on the surface against the water move forward. On reaching the anterior end, they travel over the tip of the pseudopodium and remain stationary on the substratum until the moving amoeba has passed on. They then are carried back again on to the free surface. He concluded from this that the amoeba progresses by a rolling motion which is imparted to the relatively inactive and solid ectoplasm by the streaming of the fluid endoplasm.

Grüber (1912) and Schaeffer (1917, 1921) showed conclusively that it is not the entire ectoplasm which moves, but an extremely thin layer on the outer surface of the ectoplasm. Grüber regarded this layer to be a permanently differentiated layer of gelatinous material, possibly a mucus-like coat. Schaeffer regards it as a film of liquid protoplasm which covers the more solid ectoplasm. He accounts for the progress of the amoeba by the energy in a liquid surface film. On the other hand, the micro-dissection method sustains the conclusions of Grüber that the amoeba is covered by a pellicle of relatively inactive material. This pellicle varies in toughness in different species. In *Amoeba verrucosa* it is extraordinarily tough and Howland (1923, 1924) has shown that it can be pulled and stretched by micro-needles without injuring the amoeba (Fig. 22).

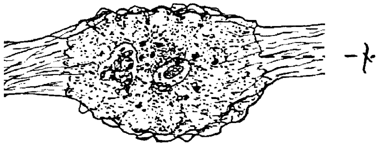

FIG. 22.—*A. verrucosa* with pellicle lifted off on both sides by needles (Howland, 1924).

In *Amoeba proteus* the pellicle is very thin and tenuous. It can be easily lifted off by injecting water beneath it. The blister-like elevation which is thus formed persists for a time, and then gradually disappears as the underlying liquid apparently diffuses out. A slight puncture causes the blister to burst and the pellicle to collapse.

The micro-dissection method also shows that the hyaline zone of ectoplasm immediately under the pellicle is usually liquid. By dark-field illumination it is optically empty (Gaidukov, 1910). Beneath this hyaline zone is the granular mass occupying the interior of the amoeba. The outer zone of the granular mass, the granular ectoplasm, is relatively solid, and merges insensibly into the fluid interior, the endoplasm. The hyaline liquid area between the granular ectoplasm and the external pellicle is not always present so that the pellicle may lie directly on the granular ectoplasm. It may, however, attain very large proportions, for example, by the application of heat (Grüber, 1911) and by micro-injection. If an amoeba with a pronounced peripheral hyaline zone be selected and an aqueous suspension of lamp black be injected into the hyaline zone, the lamp-black particles will immediately spread throughout the hyaline zone, indicating that it is a liquid. This is contrary to the generally held opinion (Schaeffer, 1920; Mast, 1923).

The relatively solid granular ectoplasm under the hyaline zone and the endoplasm are pronouncedly contractile. This is well shown by the injection of a large amount of water as described on page 263. After a preliminary dilution effect, the granular ecto- and endoplasmic mass contracts, and a fluid collects in the hyaline zone under the pellicle as if the water had been squeezed into it.

2. *Viscosity changes in the formation of the pseudopodium:*

When a pseudopodium is about to form, there occurs a localized liquefaction of the granular ectoplasm after which the freed granules pour out into the hyaline zone under the pellicle. If the hyaline zone is very wide the granules tend to spread out in it. Otherwise, the pellicle bulges locally and the granules stream into the bulge.

Frequently a pseudopodium begins by the bulging of the pellicle, after which the peripheral granular ectoplasm undergoes liquefaction, and the imbedded granules scatter and stream forward into the bulge. They flow to the extreme tip of the pseudopodium where they are caught in currents which flow back apparently immediately beneath the pellicle.

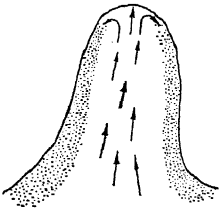
FIG. 23.—Formation of a pseudopod.

These streaming movements under the pellicle closely resemble the so-called fountain currents of Rhumbler (1914), which occur in a liquid drop whose surface tension has been locally diminished. The back flow in the pseudopodium is, however, quickly arrested by a solidifying of the flowing material as it comes into contact with the more solid material around the base of the pseudopod. This setting process builds up a semi-solid wall about a central freely flowing channel (Chambers, 1920). As the pseudopodium extends still farther the peripheral back flow at its tip continually adds solidifying material to the top of the hollow, cylindrical wall (Fig. 23). With a slowing down and cessation of the axial stream, the tip of the pseudopod solidifies, and we now have an extended arm, with a jelly-like, solid wall, which is elastic.

In attempting to explain pseudopodial formation the following points must be taken into account: (1) A protrusion of the pellicle appears with a localized liquefying of the underlying granular ectoplasm. (2) The liquefied material and the fluid endoplasm flow into the bulge which thereby becomes extended. (3) Back of the pseudopodium, the body of the amoeba, especially at its posterior end, diminishes in size and tends to exhibit surface wrinkles. This may be due to an active contraction of the granular ectoplasm compressing the endoplasm into the pseudopodium or to a more or less passive shrinkage effect produced by the streaming away of the endoplasm into the pseudopodium. (4) The endoplasm streams to the tip of the pseudopodium where it spreads and flows back as a peripheral current. The back flow is normally arrested by a peripheral solidification process. (5) The pellicle moves forward on the free surface of the pseudopodium but is stationary where the pesudopodium adheres to a substratum (Jennings, 1904).

Bütschli (1892) who studied a very fluid amoeboid organism, *Pelomyxa*, noticed the forward flow of a surface film external to the peripheral back flow. The back flow he regarded as being due to a lowering in surface tension. He comments on the extraordinary picture of two apparently contiguous but oppositely directed currents. Bütschli assumed that the external boundary of the amoeba is fluid, and was at a loss to explain the phenomenon. If, on the other hand, we regard the boundary as a more or less solid pellicle in the way that Jennings regarded the ectoplasm and, further, that this pellicle is non-adherent to the underlying protoplasmic surface film of the amoeba, we might expect that a local weakening of the film with the formation of a bulge at the anterior end, together with a contraction of the granular ectoplasm back of the tip, would stretch the pellicle and cause an adhering particle to travel forward. The variation in the movement of particles on the surface, which Schaeffer (1920) found, may be due to local variations in the viscosity of the protoplasm.

Mast and Root (1916) have presented evidence which they regard as antagonistic to the assumption that the tip of the pseudopodium is a region of diminished surface tension. They observed *Paramoecia* become divided into two pieces on being caught between the advancing tips of two opposing pseudopodia. They inferred that the pseudopodia pinched the relatively solid body of the *Paramoecium* by mechanical force. Such an assumption, however, is not necessary. If one side of a *Paramoecium* body be slightly injured with a micro-needle, the highly irritable ectoplasm at the spot of injury will often curl in to such an extent that the *Paramoecium* actually pinches itself in two. One may infer, therefore, that the pseudopodia of the amoeba observed by Mast and Root merely initiated an injury and that mechanical pressure on the part of the pseudopodia had little to do with the process. However, a somewhat similar observation was made by Lieberkühn (1870) who saw a frog's leukocyte partially ingest a red blood corpuscle, the protruding portion of which was pinched off. Even on the assumption that the tip of a growing pseudopodium is an area of diminished tension it is still possible for it to possess a certain tensile strength if we assume an investing extraneous pellicle.

3. *The existence of an antero-posterior viscosity gradient:*

In the localized viscosity changes connected with amoeboid movement, certain regions tend to maintain the more solid condition. An interesting case is shown in Figure 24, where two pseudopodia began to form at closely contiguous spots. The liquefying of the granular ectoplasm at the base of each pseudopod resulted in the partial isolation of a small mass of solid material between them. The two hyaline bulges became confluent over this mass, and the depressed region of the pellicle between the pseudopodia lifted so that the two pseudopodia merged. Sometimes the solid mass may become entirely surrounded by fluid endoplasm. It will then float in the endoplasm as an inert body, where it gradually disappears by a process suggesting that of erosion. If it is very large, however, its inertia causes it to be left behind by the streaming endoplasm. This procedure, repeated in different parts of the amoeba, finally results in the accumulation at the posterior end of the amoeba of those constituents of the protoplasmic material which are the most refractory to the liquefying process. This is possibly why pseudopodia form more readily at the anterior rather than at the posterior end. It also seems to be true that the most recently solidified regions are the ones which liquefy the most readily, a fact which is true of most colloidal solutions, e.g., they tend to lose their reversibility with age.

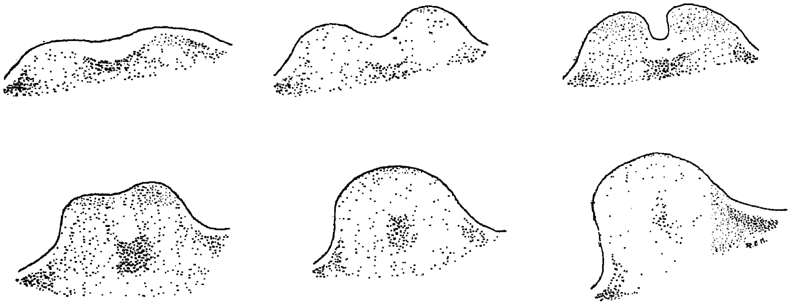

FIG. 24.—Successive stages in formation of two contiguous pseudopodia

Solidified areas in the endoplasm which are refractory to the liquefaction process can also be experimentally produced. If a small quantity of a basic dye, e.g., neutral red (cf. p. 263) be injected, the area immediately about the injection will coagulate. This coagulum tends to be left behind in the streaming of the endoplasm until it comes to lie in the posterior end (Chambers, 1920).

4. *The effect of experimentally induced changes in the solidifying and liquefying tendencies:*

The following experiments indicate that the type of pseudopodia formed and the motility of the amoeba as a whole depends largely upon the balance between the liquefying and solidifying tendencies of its protoplasm.

The balance can be shifted to the more fluid side by continued agitation of a moving amoeba. The jellying tendency being diminished, the peripheral back flow in a pseudopod will continue longer so that it spreads and widens the base of the pseudopod before the protoplasm sets. The extending pseudopod, having a larger base upon which to build, becomes broadly lobate. On the other hand, if the jellying tendency be dominant, the base of the pseudopod is quickly limited by the rapid setting of the protoplasm. The extending pseudopod then conforms itself to the narrow base, and becomes long and slender.

Broad and narrow pseudopodia can be experimentally induced. A trace of acid throws the balance to the more solid side while alkali does the reverse (Chambers, 1921*d*). The injection of a trace of hydrochloric acid into an amoeba at first increases its activity; in a few minutes, however, it forms long slender pseudopodia. The injection of sodium hydrate, on the other hand, causes the amoeba to form broad, lobate pseudopodia.

A change in the type of pseudopodia also occurs by varying the water content of the organism. This is shown by the experiments of L. Loeb (1921), who immersed the blood cells of *Limulus* in various salt solutions. In hypertonic solutions the cells produced threadlike pseudopodia, whereas hypotonic solutions caused the formation of broad pseudopodia.

The micro-injection results of the opposite effects of acids and alkalies have been recently confirmed by Edwards (1923), who made local applications of various solutions on the surface of the amoeba. He found that acids produce a local swelling followed by gelation, whereas alkalies produce a swelling without subsequent gelation.

VIII. INTERNAL CHANGES IN CELL DIVISION

The necessity of the nucleus for the life of the cell makes it obligatory for normal cell division to be accompanied by the division of the nucleus. Nuclear division, however, without subsequent cytoplasmic division is typical of a great many cells, both plant and animal, and can, moreover, be experimentally produced in some cells which are normally uninucleated (O. and R. Hertwig, 1887; R. Hertwig, 1896; J. Loeb, 1895, 1896; Boveri, 1897; and Wilson, 1901*b*). On the other hand, cells can be made to fragment independently of the nucleus only as an injury phenomenon by plasmolytic agents. Examples of such agents are electric shocks, hypertonic solutions, etc., which cause the protoplasm of a cell to contract and to round up. If there are strands extending from a central protoplasmic mass, the plasmolytic agent often causes the strands to clump into several completely isolated bodies. This, however, is purely an injury phenomenon.

Except by plasmolytic methods, there are no cases on record in which cell division has ever occurred or has ever been experimentally induced without the presence of an active nucleus or of active nuclear material.

The division of the nucleus is accompanied by important changes, which often result in a pronounced deformation of the surface of the cell, and, in some eggs (e.g., *Chaetopterus*) cleavage is normally associated with the elevation of a lobe of non-nucleated material through pronounced surface movements of the cytoplasm (F. R. Lillie, 1906).

In marine ova, eggs have been induced to divide after the egg nucleus had been removed. One must remember, however, that these eggs have undergone a maturation process during which the nuclear material of the large germinal vesicle permeates their cytoplasm. The mature egg, therefore, differs from the usual type of cell in being permeated with nuclear material, and its structurally visible nucleus is only a small portion of the original nucleus in the immature egg.

The several cases recorded in literature, therefore, in which mature eggs have been found to segment independently of the formed egg nucleus, do not vitiate the foregoing statement that nuclear material is essential for cell division. These cases are discussed on page 286.

1. *The aster:*

A radial arrangement of cytoplasmic granules about one or more centers is a feature of eggs which are preparing to undergo cleavage. The radial configuration has suggested for these structures the term "aster." Asters in the fertilized ovum, already observed in the early forties, were first associated with cell division by Fol in 1873. Most of the earlier investigators, including Auerbach, O. Hertwig, Fol, Bütschli and Strasburger, but especially Fol, suggested that the radial figure is due to centripetal diffusion currents which arrange the cytoplasmic granules in rows about a center. This view, largely based upon the fact that the central area of the aster increases as the aster develops in prominence, has been more recently upheld by Rhumbler (1898), Ziegler (1898), Morgan (1900), Wilson (1901*a*), Jenkinson (1904), Mathews (1907), and Chambers (1917*b*).

Although the aster is not apparent in all cases of cell division, it has been constantly associated with so many cases that a study of the changes which accompany it must throw light on the problem of cell division. Normally, the aster is always associated with the nucleus, in the neighborhood of which it first appears in diminutive form and from which it gradually extends in all directions throughout the substance of the egg. Under experimental conditions several asters can be made to appear in different parts of the cytoplasm in the mature egg (Morgan, 1896, 1899). Artificial parthenogenetic agents are very liable to produce multiple asters (e.g., Wilson, 1901*a*).

a) MUTUAL REPULSIONS OF ASTERS

It is significant that when several asters appear within the cytoplasm they always occupy positions which indicate a mutual repulsion as they extend in size.

Rückert (1899) found that sperm nuclei which enter the selachian egg repel one another in the animal pole area but not in the yolk region of the egg. Brachet (1910), working with frog's eggs, confirmed Rückert's observations but showed further that the repulsion is due to the formation of an aster about the head of each sperm. In the yolk region the aster forms very slowly and the sperm nuclei tend to unite before an aster appears. Brachet used the fact that the path of the sperm in the frog's egg can be readily traced by the presence of pigment granules which the sperm carries in with it from the egg's surface. Figure 25 (from Brachet) shows the paths of two sperm which chanced to enter the egg near together. After maintaining a parallel course for some time, there developed an aster about the head of each sperm, whereupon the paths diverged and the extent of divergence steadily increased with the growth of the asters.

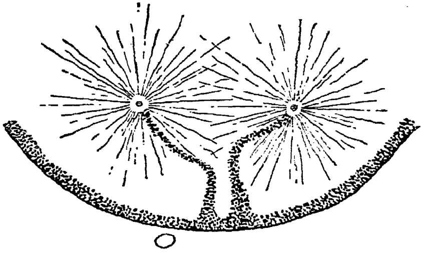

FIG. 25.—Two sperm asters in frog's egg (Brachet, 1910).

Brachet concluded from his observations that the aster is an area of coagulation, a postulate tentatively advanced by Teichman (1903), M. H. Fischer and W. Ostwald (1905), and used by Delage (1907) to explain the action of the parthenogenetic agents which he used on echinoderm eggs. Another view, however, which has been advanced is that the aster is a resultant of electrical repulsions (Lamb, 1908; Hartog, 1905; F. R. Lillie, 1909; R. S. Lillie, 1916).

b) DISAPPEARANCE AND REAPPEARANCE OF THE ASTERS

O. and R. Hertwig (1887), O. Hertwig (1890), and Wilson (1901*b*) noted that the asters are temporary phenomena within the fertilized egg and can be made to disappear completely on cooling, etherizing, or even shaking the eggs. Mathews (1907) secured similar results by suppressing oxidations. When the eggs are returned to normal surroundings, the asters reappear. Agitation of the aster with the micro-needle will also cause it to disappear temporarily (Chambers, 1917*b*).

c) THE ASTER CONTROLS THE POSITION OF THE CLEAVAGE FURROW

In normally segmenting eggs two asters appear in the egg, one at each pole of the nucleus, and the cleavage furrow always forms between the two asters. Although the aster is normally always intimately associated in its spatial relations with the nucleus, we have already seen that under certain conditions asters may appear in the mature egg far removed from the nucleus. Such asters are known as cytasters. The cytasters appear to be identical with the nuclear asters except for the absence of a nucleus (Wilson, 1901*b*). It is of interest to note also that cytasters are able in the same manner as the nuclear asters to determine the position of cleavage furrows. Figure 26 (from Wilson) shows a *Toxopneustes* egg in which cytasters had been made to appear by abnormal treatment (etherization). Notice that the cytasters, with reference to the forming furrows, occupy positions similar to those of the nuclear asters. They all lie in the protrusions of the egg.

The furrows between nuclear asters normally complete their course so as to divide the egg into blastomeres. The furrows about cytasters may do the same. Ziegler (1898) was the first to record a case of a sea-urchin egg which budded off a portion of its cytoplasm containing a cytaster but no nucleus. Wilson (1910*a*) has also shown that sea-urchin eggs which have been artificially induced to form cytasters may frequently undergo cleavage about the cytasters. These cytasters, however, were always in cells containing both a nucleus and a typical nuclear aster.

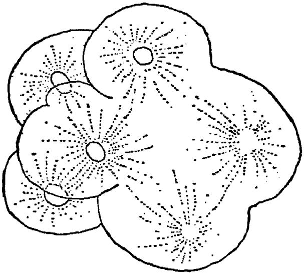

FIG. 26.—Nuclear and cytasters in *Toxopneustes* egg (Wilson, 1901*b*).

Accompanying the formation of the aster, be it a cytaster or a nuclear aster, the surface of the egg becomes distinctly mobile in the regions farthest removed from the center of the aster. This is well shown when a single aster happens to grow in size and remain in an eccentric position within the egg. Painter (1918) found that in such monaster eggs the side farthest from the aster may not only develop irregular elevations but may even bud off globular masses. It is very probable that this mobility of the egg's surface is analogous to what occurs during cleavage furrow formation (cf. p. 294). And the constant association of the aster with the mobile condition of the egg's surface strongly suggests that the aster has something to do with this condition.

If this is true, then the question arises whether the cytaster lacking a nucleus is or is not capable of producing a cleavage furrow when there is no nuclear aster present in the same protoplasmic body. McClendon (1907, 1908) claimed that this can occur. He obtained parthenogenetic development of the mature starfish egg after having removed its nucleus by removing the maturation spindle during the polar-body formation. These enucleated eggs segmented into a morula-like mass of cells, each of which contained a cytaster. Wilson (1901*b*) caused the appearance of cytasters in enucleated *Toxopneustes* eggs, but never found anything simulating a regular segmentation of the parthenogenetically treated enucleated eggs.

In the starfish egg cut in two and then subjected to a parthenogenetic agent (butyric acid), I have observed fragmentation of the non-nucleated piece.

Some of the fragments possessed cytasters, others did not. This suggests that the cytaster is not a prime factor in the budding process and that the division of the non-nucleated piece is a fragmentation caused possibly by an extremely mobile condition of the egg's surface.

A recent observation of Jollos and Péterfi (1923) also suggests this. These investigators punctured a fertilized amphibian egg with Péterfi's micro-dissection apparatus to remove one of the pronuclei. Within twenty-four hours the egg fragmented into a number of pieces without the participation of a nucleus.

d) APPEARANCE OF THE SPERM ASTER AND ITS PHYSICAL STATE

O. Hertwig (1876), Fol (1879), and Wilson (1895) showed that the aster which appears in the fertilized egg of the sea urchin commences as a diminutive radial configuration about the head of the spermatozoön within a few minutes after it has entered the egg. As the aster grows in size, there appears in its center about the sperm head a hyaline area. The growth of the aster consists in a gradual extension of its rays and an increase in volume of its central hyaline region, the centrosphere.

Coincident with the growth of the sperm aster there occurs an increase in viscosity of the egg cytoplasm. This increase in viscosity upon fertilization has been carefully worked out in the sea-urchin egg by Heilbrunn (1915, 1920, 1921) with the centrifuge method. Odquist (1922), by using Heilbrunn's method, has found the same to be true for the frog's egg.

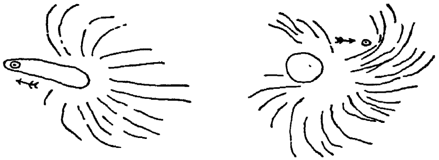

FIG. 27.—Early sperm aster in sand-dollar egg being dragged and pushed by a needle (Chambers, 1917*b*).

The connection between the increase in viscosity with the growth of the sperm aster has been shown by the micro-dissection method. If a needle be inserted through the egg into this sperm aster, one can push and roll the aster about, as if it were a viscous body lying in the fluid cytoplasm. Figure 27 shows how the early aster can be dragged about by a needle. If left untouched the growing sperm aster gradually shifts from its eccentric position until its center finally occupies the center of the egg. By this time (about 25–30 minutes after insemination), the rays have spread in all directions almost to the periphery of the egg. They are innumerable, fine, hyaline streaks in the granular cytoplasm. The hyaline region, centrosphere, in the center of the aster, is distinctly fluid, for the tip of a needle inserted into it can be moved about without meeting any resistance. The nucleus which lies within it can be pushed about with ease.

The jellied state is most pronounced in the cytoplasm bordering the centrosphere, its viscosity diminishing as one approaches the periphery of the egg. The viscous state of the aster is such that a needle moved through the aster will produce a distortion and twisting of the jellied strands of granular cytoplasm with the fluid rays between (Fig. 28).

The border of the central hyaline area is broken by the converging tips of granular cytoplasm which project into the hyaline area, Figure 29 (cf. Fig. 32).

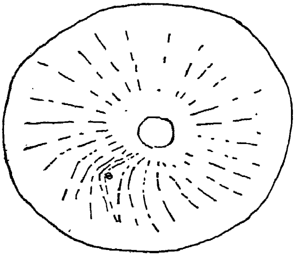

FIG. 28

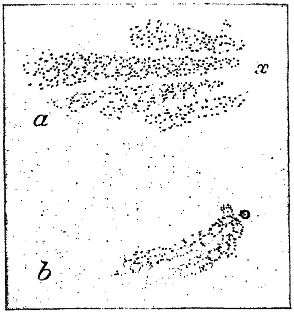

FIG. 29

FIGS. 28–29: FIG. 28.—Local bending of radiations of sperm aster by insertion of a needle (Chambers, 1917*b*). FIG. 29.—(*a*) Central ends of radial strands of aster projecting at *x* into central hyaline sphere. (*b*) Projecting tips bent by needle (Chambers, 1917*b*).

Between these projections the hyaline rays of the aster merge with the hyaline fluid of the centrosphere.

The granular cytoplasm about the centrosphere is comparatively solid. Figure 29*b* shows how the projections into centrosphere can be bent with the needle. The hyaline rays which project between the granular streaks are fluid and apparently identical with the fluid of the centrosphere.

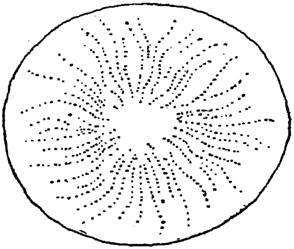

FIG. 30.—Sperm aster which has reappeared after churning egg.

Gentle churning of the cytoplasm with a needle may cause the astral configuration to disappear without producing a change in the viscosity of the cytoplasm. The centrosphere persists, however, and the rays reappear when the egg is left undisturbed. They are, however, twisted and curved (Fig. 30). If the agitation be carried farther, the cytoplasm reverts to the fluid state of the egg at the time of insemination.[1]

When sand-dollar eggs are treated with artificial parthenogenetic agents there are some modifications in the appearance of the aster which help to throw light on the aster formation in the sperm-fertilized egg (Chambers, 1921*a*). The eggs are treated with butyric acid and hypertonic sea water (cf. Loeb, 1913) and then returned to sea water. After several minutes, the first sign of a change consists in the appearance of faintly defined hyaline areas near the center of the egg. Within a few minutes these hyaline areas coalesce to form a central clear area of about one-tenth the diameter of the egg. The egg nucleus lies close to or within this area. Gradually rays begin to appear in the cytoplasm about the area. These rays become more numerous and more pronounced until the entire egg is occupied by a large aster which corresponds exactly with the fully developed sperm aster of a normally inseminated egg. The visible phenomenon peculiar to the parthenogenetic egg consists in the appearance of vacuoles which unite to form a hyaline center *before* the astral rays appear. In the sperm-fertilized egg the whole process is more rapid; radiations appear immediately about the sperm head, and the accumulation of the hyaline substance is from the very start apparently through the agency of the raylike channels of the growing aster. In the parthenogenetic egg, the jellying process is apparently very slow, and the separating out of a liquid takes place before the cytoplasm is stiff enough to exhibit channels through which the liquid flows to the center. The liquid first collects into several clear areas, and, when the parthenogenetic treatment is at its optimum, the areas fuse into one body about which an astral configuration subsequently appears.

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[1] The union of the pronuclei in relation to the viscosity of the sperm aster is under investigation. Some features of it in relation to the viscosity of the aster have already been described (Chambers, 1917*b*).

2. *The amphiaster:*

The maturation divisions and the segmentation of the egg are typically preceded and accompanied by the development of two asters, one at each pole of the nuclear spindle. The two asters and the spindle between them constitute the amphiaster.

In *Cerebratulus*, a nemertine worm, the maturation spindle of the egg occupies only a very small portion of the cytoplasm of the egg (Fig. 31*a*). The jellying process involved in the formation of the amphiaster about this spindle anchors the peripheral aster to the cortex of the egg while the aster at the other end projects freely into the deeper and more fluid portion of the egg cytoplasm. By seizing this end with a needle the entire figure may be stretched and distorted (Fig. 31*b*), but cannot be dislodged from the egg periphery without becoming disorganized. As soon, however, as the spindle elongates and divides in the middle the more deeply lying polar aster surrounding the egg nucleus is set free and can be carried by the needle to any position within the fluid cytoplasm of the egg (Chambers, 1917*b*). In this new position the second amphiaster is formed, with the result that the second polar body is produced at a distance from the first. Conklin (1917) secured similar results in *Crepidula* eggs by subjecting them to centrifugal force during the various stages of polar body formation.

In some species, e.g., the starfish and the mollusk *Cumingia*, the astral configuration about the sperm nucleus in the fertilized egg is from its inception a double structure. The rays center about two spots at one side of the sperm nucleus to constitute the two polar asters of the so-called amphiaster. At first very diminutive, the two asters increase in size with an extension of their rays and an enlargement of the hyaline area (centrosphere) in the center of each aster. This increase in size and extent is accompanied by an increase in the distance between their centers, together with a lengthening of the hyaline nuclear "spindle" which extends between their centrospheres (Mathews in Wilson and Mathews, 1895).

In the eggs of other species, e.g., the sea urchin and the sand dollar, the aster about the sperm head is a single structure, the so-called sperm aster. At its maximum growth it occupies practically the entire egg, and it is this aster which has been made the principal object of study in the preceding subsection. While it is present the viscosity of the egg is high. Shortly before the cleavage of the egg it becomes indistinct (Wilson, 1895) and the viscosity of the cytoplasm decreases (Chambers, 1917*b*).[1]

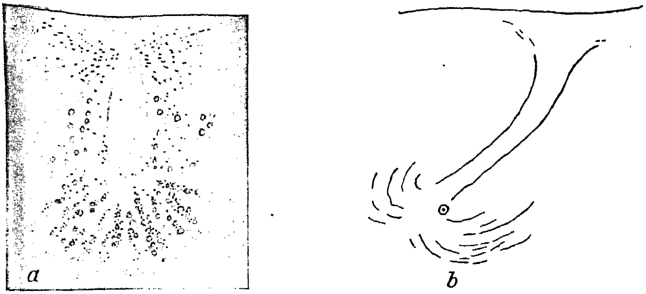

FIG. 31.—(*a*) Maturation spindle in *Cerebratulus* egg. (*b*) The spindle stretched by a needle (Chambers, 1917*b*).

This change in viscosity from that of the sperm-aster stage is so gradual, however, that the radial arrangement of the cytoplasmic granules tends to persist. However, the slightest agitation of the cytoplasm with the needle destroys this configuration entirely. This period is succeeded by a reappearance of rays grouped about the two poles of the nucleus to form two asters instead of one as before. This constitutes the amphiaster.[2]

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[1] The significance of the single sperm aster is not understood. The suggestion has been made that it indicates an abortive attempt on the part of the egg to divide.

[2] Regarding the initiation of the amphiaster, all we can say is that there exists in the cell a mechanism which periodically starts up two centers or foci as it were (the centrosome hypothesis of cytologists) which collect the materials of the mother-cell into two groups to constitute the two daughter-cells. For a somewhat hypothetical but suggestive discussion of a centrosomal force, the reader is referred to a recent paper by Cannon (1923).

From this time on the procedure in both types of eggs is the same.

Figure 32 is a side view in optical section of the inner region of a living sand-dollar egg in the amphiaster stage. The hyaline rays, extending from the hyaline centrospheres, give to the granular cytoplasm a streaked appearance which is finer than that represented in the drawing. The presence of the hyaline nuclear spindle which extends through the equatorial region of the egg between the two centrospheres of the polar asters makes the astral configuration incomplete in this region. Around the spindle the rays of the two opposite asters meet at the equator of the egg, and sometimes give the appearance of crossing one another.

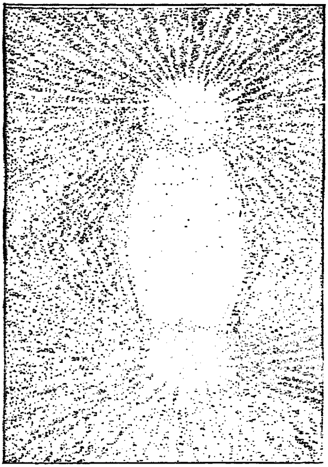

FIG. 32.—Amphiaster of sand-dollar egg (Chambers, 1917).

Shortly before division of the egg the amphiaster attains its maximum development and occupies the entire substance of the egg. It is at this time that the egg elongates preparatory to cleavage.

The amphiaster is highly susceptible to injury and its radial configuration can be easily made to disappear on inserting the micro-needle and agitating the cytoplasm.[1] If, however, this operation be carefully done, the amphiaster can be distorted within the egg and its rays can be made to twist and curve (Fig. 33, p. 293). This twisting of the rays has already been noted by Morgan (1910), Spooner (1911), and Conklin (1917), who produced distortion effects of the nuclear spindle and of its astral rays by means of the centrifuge method.

The disappearance of the rays of the amphiaster by agitating with the needle is accompanied by a decrease in the viscosity of the cytoplasm. The nuclear spindle can then be moved out of the center of the egg. If the egg be left undisturbed, rays reappear about the two poles of the spindle which, with the increase in size of its polar asters, gradually moves back to a more central position. If spindle be moved to one side of the egg and held there the polar asters will develop in an eccentric position. Figure 34 (p. 293) shows such a case. In a short time the amphiaster developed about it, producing a bulge on the surface of the egg. Figure 34*a* is a polar view and Figure 34*b* a partial view of the early amphiaster in a somewhat later stage. Note that one of the asters lies in the bulge which has increased in size. It is possible also to cause the disappearance of only one of the polar asters. Figure 35 shows such a case in which one aster only was destroyed by the needle while the other persisted. In a few seconds the dissipated aster developed again, and normal cleavage ensued.

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[1] The centrifuge method is good for a study of stable variations in viscosity but must be unsuited for detecting delicately balanced states which easily reverse upon agitation. This may be why Heilbrunn (1921) found no increase in viscosity during the development of the amphiaster. However, just before and during cleavage, when the amphiastral figure is complete, Heilbrunn noted an increase in viscosity. This accords with my results (1917*b*, 1919). The inability of Ödquist (1922) to find this is, as he suggests, due possibly to the fact that the amphiaster, in the frog's egg, occupies a very small part of the egg substance.

The further development of the egg consists in a lengthening of the amphiaster by a moving apart of its two polar asters whose rays now extend almost to the periphery of the egg. The nuclear spindle between the poles also lengthens, and the egg changes from a spherical to an ovoid shape. O. Hertwig (1876) called this the karyokinetic lengthening of the egg. Simultaneous with this the granular cytoplasm in the equator of the egg loses the streaked appearance which it hitherto possessed by the extension into it of the astral rays (cf. Fig. 32, p. 291). There is considerable evidence for assuming that the fading out of the rays in the equatorial region is accompanied by a distinct decrease in viscosity of the cytoplasm.

Aside from the observations of several investigators (von Erlanger, 1897; Conklin, 1899; Spek, 1918; Chambers, 1917*b*) on the existence of a centripetal flow of granules within the equatorial zone, we have additional evidence from the results of cutting a segmenting egg diagonal to its cleavage furrow (Chambers, 1919). An example of such an operation is shown in Figure 36. The cleavage furrow which had started before the cut was made continued its original course so that each piece of the egg pinched off a non-nucleated fragment which normally would have belonged to the other blastomere. This procedure is intelligible on the basis of assuming that the two semi-solid asters have become separated by a more fluid zone into which the furrow sinks. If, however, the egg be roughly handled during the cutting process (Fig. 37*a*, *b*), the amphiaster entirely disappears and all of the protoplasm of the egg on each side of the cut merges into a single fluid mass. The original furrow then becomes obliterated, and when a new amphiaster appears for the next cleavage (Fig. 37*c*), we find that it occupies a symmetrical position in each of the pieces produced by the cut, and cleavage takes place through the middle of each piece.

A suggestion has recently been made (Gray, 1922) that the deformation in the dividing egg is analogous to the change produced in a liquid drop by a steadily elongating column of solid material within. This cannot be true, because at the very time that the egg elongates a liquefaction of its equatorial zone takes place. Dissection with the needle also indicates that the nuclear spindle is a fluid of very low viscosity so that it is difficult to imagine that the lengthening spindle can push the polar asters apart (cf. pp. 272, 276).

The elongation of the egg may be accounted for by the collecting and stiffening of the cytoplasmic material about the two centers of the polar asters. The combined diameters of the two completed asters being greater than the original diameter of the egg a deformation of the spherical egg must result

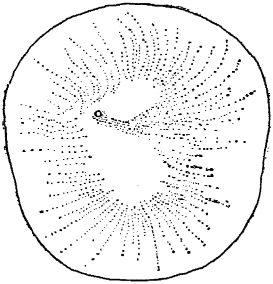

FIG. 33

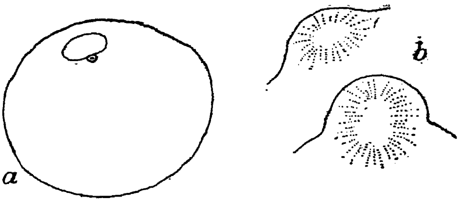

FIG. 34

FIGS. 33–34: FIG. 33.—Amphiaster in sand-dollar egg being distorted by needle. FIG. 34.—(*a*) Nucleus of sand-dollar egg pushed to periphery of egg. (*b*) Formation of amphiaster about dislocated nucleus.

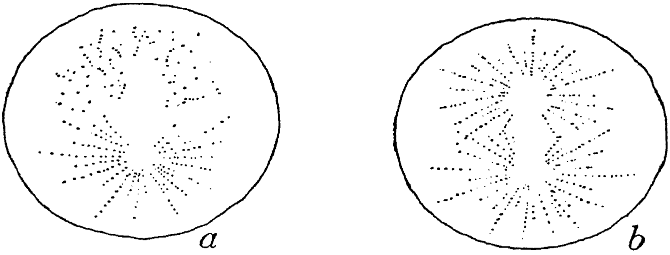

FIG. 35.—(*a*) Amphiaster egg with one polar aster destroyed by needle. (*b*) Same egg a few seconds later.

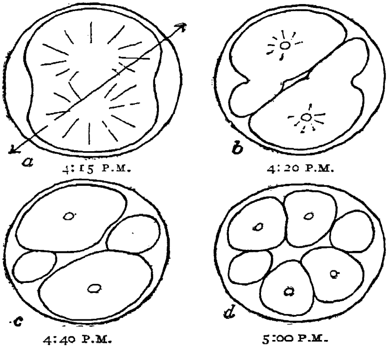

FIG. 36

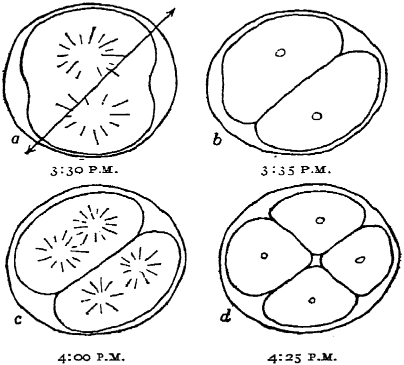

FIG. 37

FIGS. 36–37: FIG. 36.—Effect of cutting without disappearance of amphiaster (Chambers, 1919). FIG. 37.—Effect of cutting with disappearance of original amphiaster (Chambers, 1919).

(Chambers, 1919). Together with this we must take into account important surface changes which are discussed in the following subsection.

The two asters of the amphiaster are fully maintained after the completion of cleavage, and, in the absence of undue pressure from without, each blastomere tends to become spherical. This is best seen in an egg which has had its investing fertilization membrane removed prior to cleavage (Fig. 38*a*). The removal from the fertilization membrane does not entirely free the blastomeres, for they still remain surrounded by a delicate pellicle (cf. p. 254). This pellicle, however, is not strong enough to distort them as long as the asters persist. With a needle the aster in one of the blastomeres can be destroyed, whereupon the blastomere lacking the aster at once flattens against its neighbor (Fig. 38*b*). As soon as the aster reappears the blastomere rounds up again.

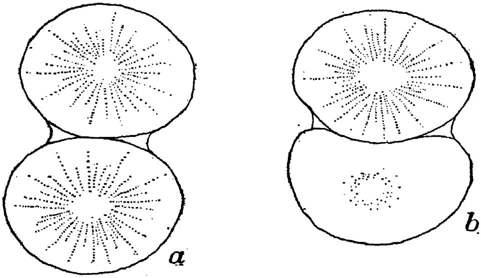

FIG. 38.—(*a*) Sand-dollar egg divested of its fertilization membrane and in the two-celled stage. (*b*) Aster in one blastomere dissipated by needle.

If left undisturbed, both asters eventually fade out and both blastomeres flatten against one another.[1] Evidently the cell, during mitosis, is in a state of internal tension which at least in the ovum is associated with an increased viscosity in the form of the astral configuration. A lowering of the viscosity by mechanical agitation causes the aster to fade out, and the cell then resumes the original plastic state.

IX. SURFACE CHANGES IN CELL DIVISION

1. *Mobile state of the surface:*

Aside from internal changes concerned with the development of the amphiaster, there are important surface changes which have a distinct bearing on cell division. Flemming long ago noted that irregularly shaped cells tend to round up during mitosis. This is apparently accompanied by an increased internal tension, for the cell swells as it rounds up even when it is crowded in among other cells.

In echinoderm eggs a surface change becomes appreciable after fertilization. The unfertilized egg is somewhat irregular in contour, and tends to possess a jellied cortex of appreciable thickness. As soon as it is fertilized it rounds up, and with the micro-needle it is possible to detect a decrease in viscosity.[1] This decrease in viscosity of the egg cortex can also be noted by the increase of Brownian movement in dark-field illumination.

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[1] The tensile strength of the surrounding pellicle varies in different eggs. In the sea-urchin egg it is strong enough to hold the blastomeres close together even when the asters are still present. In the starfish egg it is so weak that the blastomeres round up easily and would not remain together except for the surrounding vitelline or fertilization membrane (Chambers, 1923*a*).

When the amphiaster is fully developed so that the radiations of the asters almost reach the periphery of the egg, the decrease in peripheral viscosity is more noticeable both on dissection and by the dark-field method. In addition to the increased fluid state a peripheral flow becomes appreciable which, according to all observers who have described it, is directed toward the equatorial region of the cell.

Probably the first to suggest the existence of a movement toward the equator of a dividing cell was Zimmerman (1890), who described the accumulation of pigment granules in the equator of dividing pigment cells in the frog's skin. Nusbaum (1893) found a similar phenomenon in the embryonic endoderm cells of the frog. Flemming (1891) also suggests a differentiation of materials during mitosis, for he found that the equatorial region of dividing epithelial cells differs from the polar regions in staining more heavily with osmic acid.

The migration of peripheral granules to the equator and their accumulation in the walls of the cleavage furrow have been found to occur in the ova of *Ctenolabrus* (J. Loeb, 1895) and of *Polychoerus* and *Aphanistoma* (Gardiner, 1895). Erlanger (1897) observed a distinct flow of cytoplasmic granules in the more rapidly dividing eggs of various nematodes. Conklin (1899, 1902) inferred the existence of vortical movements from the displacement of the astral figures in *Crepidula* during the formation of the furrow. Andrews (1897), Fischel (1906), and McClendon (1911) described the migration of the pigment granules to the equatorial region in the sea-urchin egg, and I (1917*b*) have noted the inward movement of granules from the surface of the equatorial region in the sand-dollar egg. However, the most striking observations are those of Spek (1918), who was able, by the application of warmth, to render more appreciable the extensive surface currents in the dividing nematode eggs (Fig. 39). In the nematode egg, the sinking in of the cleavage furrow does not occur simultaneously all around the equator of the egg, but alternates first on one side and then the other. As it deepens, peripheral currents flow from all sides to the region of the furrow and down its walls (Fig. 39*a*, *b*, and *c*, p. 296).

Spek, from observations on a variety of nematode eggs, concluded that a correlation exists between the rapidity of cleavage and the rate of the peripheral flow which, presumably, is a function of cytoplasmic viscosity.

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[1] The mobile state of the surface is somewhat masked in the fertilized echinoderm egg by the investing delicate pellicle, the so-called hyaline plasma layer (cf. p. 254). Just (1922) has recently revived the idea that the so-called hyaline layer is an integral part of the cytoplasm and is of peculiar significance in the formation of the cleavage furrow. This is not true, because dissection shows that it is sloughed off as a thin membrane a few minutes after fertilization and can be dissected away (Chambers, 1923*a*) without affecting subsequent normal cleavage.

The existence of such a correlation is strikingly apparent on contrasting the nematode egg with that of the sea urchin. In the very fluid egg of *Rhabditis pellio,* Spek found that cleavage occurs in less than two minutes, and the surface currents associated with it are distinctly appreciable to the eye. In the much more viscid sea-urchin (*Arbacia*) egg, it takes ten to fifteen minutes for the cleavage furrow to complete its course, and the movement even of the prominent pigment granules in the periphery is too slight to be noticeable except by recording their relative positions from time to time. Figure 40 is a sketch of a part of the cleavage furrow in the sea-urchin egg. The pigment granules indicated in the walls of the furrow had moved there during the seven minutes that they were under observation.

It is significant that all the observations on record regarding the existence of a flow in the normal dividing cell agree that the flow is a superficial one and always so directed as to cause an appreciable accumulation of visible granules in the walls of the deepening furrow. The direction of the flow toward the equator makes impossible any explanation on the basis that cell cleavage is analogous to the mechanical separation of a droplet into two parts. This is Gray's (1922) interpretation based on Plateau's experiment of suspending an oil drop on two metal rings and moving the rings apart until the drop breaks in two. When a fluid drop breaks in two by being pulled apart, the curvature of the intervening bridge of fluid and the direction of the streaming movement in the narrowing bridge are quite different from that which obtains in the dividing cell. If a fluid droplet of egg cytoplasm be stretched between two needles, the fluid cytoplasm in the intervening portion will flow *from* the middle toward the needles as these are moved apart until the droplet finally breaks in two. Nothing like this occurs in the dividing cell.

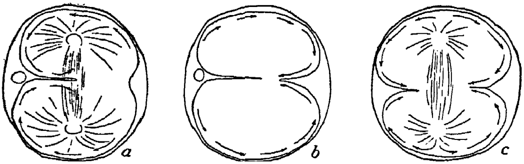

FIG. 39

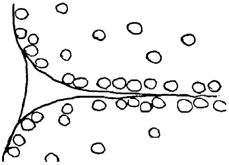

FIG. 40

FIGS. 39–40: FIG. 39.—Nematode egg segmenting, (*a*) and (*b*) one minute apart. (*c*) Egg with symmetrically segmenting furrow (Spek, 1918). FIG. 40.—Pigment granules on periphery of sea-urchin egg collecting in walls of cleavage furrow.

A more plausible suggestion to account for cleavage is that of Bütschli's. Bütschli (1876) postulated that differences in surface tension are produced by the growing asters as soon as they extend to the egg's surface. Surface changes actually do occur in eggs with fully extended monasters, for the contour of the surface then frequently exhibits irregularities which sometimes simulate pronounced amoeboid movements. The existence of these irregularities has been described by many as an argument in favor of the astral radiations being contractile fibers which exert a pull on the egg's surface. With the micro-dissection needle, however, one can show that the aster has no solid fibers anchored to the surface of the cell but consists of cytoplasm, the viscosity of which diminishes toward the periphery (Chambers, 1919).

In the amphiaster egg, with two eccentrically placed polar asters, the extension in size of the asters, according to Bütschli's idea, would first affect the two opposite polar surfaces of the egg. This would set up a difference between the polar and equatorial regions of the egg.[1] One evidence of such a difference is the peculiarly directed peripheral currents in the dividing egg. Additional evidence is furnished by Just (1922), who found that amphiaster eggs tend to burst more easily at their poles than at their equator when the eggs are made to swell by immersion in hypotonic sea water.

The micro-dissection method offers still more evidence by showing that the polar and equatorial regions of the dividing egg differ in their state of fluidity. A tear made by the needle in the polar region tends to gape, and closes very slowly, whereas a tear in the equator closes up immediately. The more pronounced fluid state of the equatorial region can also be detected in the dark field, especially in centrifuged sea-urchin eggs.

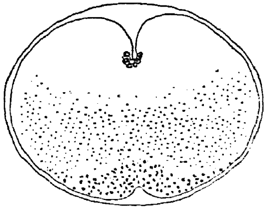

FIG. 41.—Centrifuged sea-urchin egg cleaving.

The eggs are first centrifuged and then fertilized. The centrifugal action masses the heaviest cytoplasmic granules to one side of the egg, while the opposite side becomes filled with a hyaline fluid capped by a small collection of globules (cf. p. 245). If an egg be selected whose nuclear spindle lies parallel to the plane of stratification of the cytoplasmic contents, the cleavage furrow will pass through the dense granular area on the one side and through the very fluid zone on the other (Fig. 41). On examining such an egg in the dark field, some time before cleavage begins, no Brownian movement can be detected in the heavy, dense granular area. A few seconds before the appearance of the furrow a distinct but localized Brownian movement starts up at the spot where the furrow later sinks. In connection with this, it is significant to note that the furrow on the opposite, practically liquid, side of the egg, sinks in more rapidly.

2. *The protoplasmic surface film in its relation to the formation of the cleavage furrow:*

A striking feature regarding the walls of the cleavage furrow is the fact that although in intimate contact they remain distinct, even when freshly formed.

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[1] In tissue culture cells, where no aster is evident, Burrows (1913) found that the mobility of the cell surface is distinctly increased in those regions which are approached by the two halves of the dividing nucleus.

The rapidly acquired non-coalescent property of protoplasmic surface films is discussed on page 258. The following experiment strikingly illustrates this property. If a segmenting sand-dollar egg be compressed so that it is flattened out and closely pressed against its fertilization membrane, the cleavage furrow will nevertheless cut through the equator. The compression on the egg prevents the blastomeres from rounding away from one another as they would under normal circumstances, so that the walls of the deepening furrow are in intimate contact almost from the very start. Even under these conditions, the egg divides successfully into two completely separated blastomeres.

This knife-edge-like type of furrow formation, although not normal in echinoderm eggs, is frequently met with in nature (e.g., ctenophore eggs, Ziegler, 1903). A close study of this phenomenon gives us some insight into the way in which the non-coalescent property develops in the protoplasmic surface film. The furrow always starts as a pit with non-contiguous walls. With the deepening of the furrow the walls, behind the advancing tip, come into intimate contact and leave a rounded space of noticeable size at the extreme tip of the advancing furrow (Ziegler, 1903; Yatsu, 1912). This space maintains itself even when the sand-dollar egg is kept under extreme compression (Fig. 42*a*). The presence of this space at the tip of the deepening furrow causes the bridge connecting the two halves of the egg to be of considerable length (Fig. 42*b*). With the progress of the furrow, the bridge narrows until it breaks in the middle (Fig. 42*c* and *d*). The bases of the broken bridge draw back into their respective blastomeres, leaving an oblong space (Fig. 42*e*), which persists for a time before it is obliterated by the approach of the opposite surfaces of the two blastomeres. This procedure indicates that the mechanism of cell division is so ordered as to give the walls bordering the cleavage furrow an appreciable time to acquire a non-coalescent property before they finally come into contact.

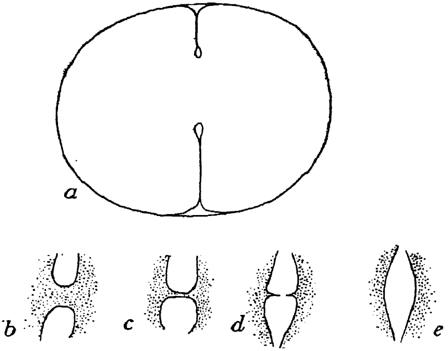

FIG. 42.—(*a*) Sand-dollar egg cleaving under compression. (*b*)–(*e*) Details to show narrowing and breaking through of bridge by advancing tips of furrow.

3. *Summary of the physical changes in the cytoplasm during egg cleavage:*

1. The nucleus of a cell may undergo mitosis regardless of the physical state of its cytoplasm while, on the other hand, the division of the cell is entirely dependent upon factors intimately associated with its nucleus. These factors influence the physical state of the cytoplasm.

2. With the formation of the nuclear spindle each pole of the spindle becomes a center about which the cytoplasm of the egg takes on an astral configuration with an accompanying increase in viscosity. The viscosity of each polar aster is at its highest about the borders of its central hyaline sphere, and diminishes toward its periphery so that both the surface of the egg and the equatorial region between the two polar asters are distinctly fluid.

3. The mitotic elongation of the egg is apparently an outcome of the growth in size of the two asters of the amphiaster, together with surface changes which produce a difference in the state of the polar regions of the egg from that of the equator. This change in state induces surface currents which travel from the poles to the equator and from thence inward. The rate of this flow varies with the viscosity of the cytoplasm.

4. When cleavage is imminent the entire periphery of the egg beneath its extraneous membranes is a flowing fluid. Within the egg the two growing asters of relatively high viscosity are separated from each other by a fluid equatorial zone.

5. The formation of the cleavage furrow depends not only upon the fluid state of the equatorial region into which it sinks, but also upon the presence of the polar asters.

6. A time factor is involved in the development of a peculiar property which prevents the walls of the cleavage furrow from coalescing when contiguous. The existence of this time factor suggests that this property of not coalescing with other cell surfaces is due to the concentration in the surface of certain substances.

X. FACTORS WHICH INHIBIT CELL DIVISION

The division of a cell can readily be stopped by many external agencies without inhibiting the division of its nucleus (cf. O. and R. Hertwig, 1887; O. Hertwig, 1890; Loeb, 1892; Demoor, 1894; Norman, 1896; Boveri, 1897; E. B. Wilson, 1901*b;* R. S. Lillie, 1903).

On the other hand, cytoplasmic division depends absolutely upon certain factors which are intimately associated with the nucleus. These factors can be nullified by a variety of external agencies. For example, cells which possess asters under normal conditions absolutely depend upon the presence of the aster for cytoplasmic division. The asters may be suppressed by such agents as cold, ether, oxygen deficiency, hypertonic and hypotonic electrolyte solutions, ammonia, and even by mechanical agitation. The nucleus, nevertheless, divides and, when the cells are returned to normal conditions, asters reappear either immediately or when the nuclei undergo their next division. The result is that the cell tends to divide into as many cells as there are nuclei.

In general, we may safely assume that nuclear division is practically independent of the state of the cytoplasm, so long as the cell is alive. Division of the cell, however, depends entirely upon the physical state of its cytoplasm.

Leaving out of consideration the chemical action of inhibiting agencies we may summarize as follows four possible physical effects which inhibit cell division: (*a*) inability of the walls of the cleavage furrow to develop a non-coalescent property; (*b*) an increase in cytoplasmic viscosity sufficient to impede the surface movements necessary for furrow formation; (*c*) a decrease in cytoplasmic viscosity sufficient to nullify the effects of differences in the state of the cytoplasm in different regions of the cell; (*d*) suppression of the aster.

1. *Condition of the wall of the cleavage furrow:*

Normally, the protoplasmic surface film very rapidly acquires a non-coalescent property. Under certain conditions, however, the property may be too weakly developed to prevent coalescence. Loeb (1896), for example, found that the lack of oxygen will cause the walls of the cleavage furrow to coalesce almost as fast as the furrow forms. This has also been done by abnormal heat (Driesch, 1893) and by immersing cells in non-electrolyte solutions (R. S. Lillie, 1903). The need of a specific cytoplasmic material to maintain the walls of the cleavage furrows is indicated in Figure 5 (p. 256), which shows the ineffectual attempts of an endoplasmic sphere to segment (cf. Chambers, 1921*b* and *e*).

2. *Inhibition due to an increase in cytoplasmic viscosity:*

Demoor (1894) in a series of experiments on cells possessing active streaming movements (staminal hair cells of *Tradescantia*, leukocytes, and myxomycetes plasmodia) found that any agent which stops the streaming movements prevents cell division. In the *Tradescantia* stamen hair the nucleus divides but no cellulose wall is deposited in the equator, and in the amoeboid cells which he studied, nuclear division occurred without subsequent cleavage of the cell.[1]

Heilbrunn (1920) with the centrifuge method found that hypertonic sea water increases the cytoplasmic viscosity of the sea-urchin egg. This increase in viscosity can also be noted, not only with the micro-dissection needle, but also by observing the cessation of Brownian movement in the dark field.

Sea-urchin eggs, in the late amphiaster stage and elongated preparatory to cleavage, were placed in hypertonic sea water ($2\frac{1}{2}$ NaCl plus 50 c.c. sea water). The eggs shrink somewhat with an increase in viscosity, and all Brownian movement ceases. On return to sea water, Brownian movement begins again, and normal cleavage follows.

The same physical effect occurs when late amphiaster eggs are placed in $2\frac{1}{2}$ per cent ether solution in sea water. All Brownian movement ceases within one to two minutes, and coincidently the cleavage process comes to a standstill (Fig. 43). On dissection, the eggs are found to be distinctly stiffened, and can be cut into angular pieces. If within five minutes the eggs be returned to

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[1] Demoor's results on inhibiting division of *Tradescantia* cells by stopping the streaming of the protoplasm has been questioned by Samassa (1898) and by Ewart (1902).

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normal sea water, Brownian movement starts again and the eggs divide in the normal manner.

In lower percentages of ether ($1\frac{1}{2}$–2 per cent), Brownian movement is retarded without being stopped, and cleavage is delayed. When cleavage takes place, wrinkles tend to appear in the region of the cleavage furrow (Fig. 44), and small pieces may pinch off as the furrow deepens. This impairment gives one the impression that the extreme surface of the egg had been rendered too stiff to conform with the peripheral flow to the equator, where wrinkles result as a consequence. A crude analogy would be the wrinkles on the cooling surface of flowing molten lava.

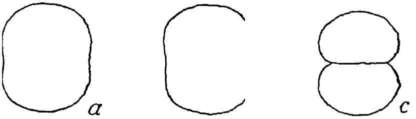

FIG. 43.—(*a*) Cleaving sea-urchin egg placed in $2\frac{1}{2}$ per cent ether. (*b*) Egg replaced 5 minutes later into sea water, where it divided (*c*).

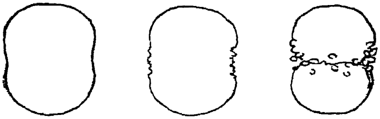

FIG. 44.—Sea-urchin egg cleaving in 2 per cent ether.

3. *The effect of a decrease in cytoplasmic viscosity:*

Ether in sufficiently low concentrations apparently tends to diminish cytoplasmic viscosity (cf. p. 250). Heilbrunn (1920) claims to have inhibited cleavage of the sea-urchin egg in this way. Weber (1922), claims to have stopped division of the *Spirogyra* plant cell by exposure to low concentrations of ether which, he found, increased the protoplasmic streaming movements. In the sea-urchin egg I was unable to stop cleavage by ether except with concentrations which decidedly increase the cytoplasmic viscosity.

On the other hand, hypotonic sea water (50 parts sea water plus 50 parts tap water) diminishes the viscosity by swelling the egg, and stops cleavage. Late amphiaster eggs with the cleavage furrow just beginning (Fig. 45) were placed in the hypotonic solution. The eggs swell and the furrow becomes obliterated. The pigment granules (indicated by black dots in the figure) which had been in the furrow now form a ring about the swollen equator. The cytoplasm of the eggs, when viewed in the dark field,

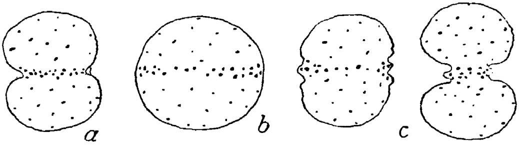

FIG. 45.—(*a*) Segmenting sea-urchin egg. (*b*) Effect of immersion in hypotonic sea water. (*c*) Continuance of cleavage on return to sea water. Pigment granules shown diagrammatically as dots.

exhibit extremely active Brownian movement of its granules. After half an hour the eggs were returned to normal sea water. The Brownian movement slowed down as the eggs shrank to their original size. The cleavage furrow re-formed, and in many cases divided the eggs completely in two.

4. *Suppression of the aster:*

Inhibition of the cleavage process can also be procured by thrusting a microneedle into the amphiaster egg and moving the needle about until the asters disappear. The entire cytoplasm of the egg is thereby brought into a uniform fluid state. The localized surface differences are obliterated, and the egg rounds up. When the agitation is stopped the amphiaster reappears and normal cleavage ensues.

We must realize, however, that asters are suppressed not only by agents whose specific action is to decrease cytoplasmic viscosity but also by those which increase it. The precise effect of these agents on the aster requires further investigation. We must, at present, assume that the aster is not only a specific gelation phenomenon but depends also upon the existence of radially converging streaming movements. If this be true, and if we assume that all inhibiting agents stop cytoplasmic streaming movements, then we can readily understand how an abnormal increase or a decrease in viscosity may cause the aster to disappear.

XI. BIBLIOGRAPHY

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SECTION VI

CYTOLOGICAL CONSTITUENTS—MITOCHONDRIA, GOLGI APPARATUS, AND CHROMIDIAL SUBSTANCE

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CYTOLOGICAL CONSTITUENTS—MITOCHONDRIA, GOLGI APPARATUS, AND CHROMIDIAL SUBSTANCE

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I. MITOCHONDRIA[1]

1. *Discovery:*

Although it is customary to trace back our knowledge of mitochondria to the brilliant researches of Altmann at Leipzig between 1880 and 1890, they were certainly seen and imperfectly described by other workers before him, notably by Flemming. Some of the bodies called "Interstitial Körner" by Koelliker, "Neurosomen" by Held, and "Cytomicrosomes" by Strasburger were clearly of mitochondrial nature. Other pioneers in cytology must also have observed them (von Brunn, L. and R. Zoja, and J. Arnold). Their relation to the chromidial substance and to the Golgi apparatus (first detected at about the same time) occupied much of the attention of the earlier workers in this specialized field. The cell seemed already to them so complicated that they were naturally loath to accept the occurrence of two additional and morphologically independent cytoplasmic constituents without convincing proof. The interesting discussion which arose is well summarized by Duesberg (1912). Thus, it has only been during the past thirty-five or forty years, slowly, with gradual improvements in technique, that the distinctive characteristics of mitochondria have come to light. We are now entering upon a period of experimentation.

2. *Definition:*

Mitochondria are composed of material exhibiting the following general properties:

a) It is of rather low refractive index but with care may be seen to occur in living unstained cells in the form of granules, rods, and filaments, and occasionally of networks, which vary in size and shape. There is some evidence that its visibility increases slightly as the cells are being studied.

b) It gives a characteristic color reaction when Janus green B (*diethylsafraninazodimethylanilin*) is applied in a solution as weak as 1:500,000. At first it assumes a bluish-green color, then on reduction, turns pink (*diethylsafranin*), and finally it bleaches to the leucobase. The delicacy of the reaction is shown by the fact that Janus green (Grübler) and Janus green C will not stain mitochondria specifically, though these dyes differ only in the substitution of an H_2 and $(CH_3)_2$ group in place of the $(C_2H_5)_2$. See page 275.

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[1] The term "mitochondria" (thread granules) is derived from the Greek μίτος, a thread, and χόνδρος, a grain. Synonyms: chondriosomes, plastosomes, and about fifty other terms invented to indicate theoretical interpretations, morphological characteristics, physical consistency, and a variety of other observed and supposed properties. The problem of terminology is discussed by Duesberg (1919) and Cowdry (1921).

c) It is very soluble in alcohol, acetic acid, and other similar reagents, and in fixed tissues it may be stained by the methods of Altmann, Benda, Bensley, and others.

3. *Technique:*

A useful summary of methods used for the demonstration of mitochondria will be found in the last edition of Lee's *Vade-mecum*, edited by Gatenby (1921). For the study of living cells, Janus green is most helpful, although other dyes have been found which will color the mitochondria more or less specifically (Cowdry, 1918; Evans and Scott, 1921). It is essential that the Janus green shall have the composition already indicated. It should be applied in salt solution in a dilution of about 1 : 10,000 or 1 : 25,000. Bearing in mind the fact that it penetrates very poorly, it must be brought into very intimate contact with the cells either by carefully teasing them apart or by injecting the fluid through the blood vessels. To make permanent preparations, the formol-bichromate and iron hematoxylin method of Regaud is recommended. The fixative penetrates well and the stain is permanent. Good contrast coloration may be obtained by using fuchsin and methyl green in place of iron hematoxylin, as advised by Cowdry (1918). It is important that small pieces of tissue not more than 3 mm. in thickness be used. The technique is not really difficult, but those who have had no experience in cytology cannot expect to be immediately successful. A little experimentation is necessary. In the matter of interpretation it must be remembered that imperfect preparations will often convey a false impression of a reduction in the amount of mitochondria and of the mitochondria being more granular in shape than they actually are in the living tissue.

4. *Morphology:*

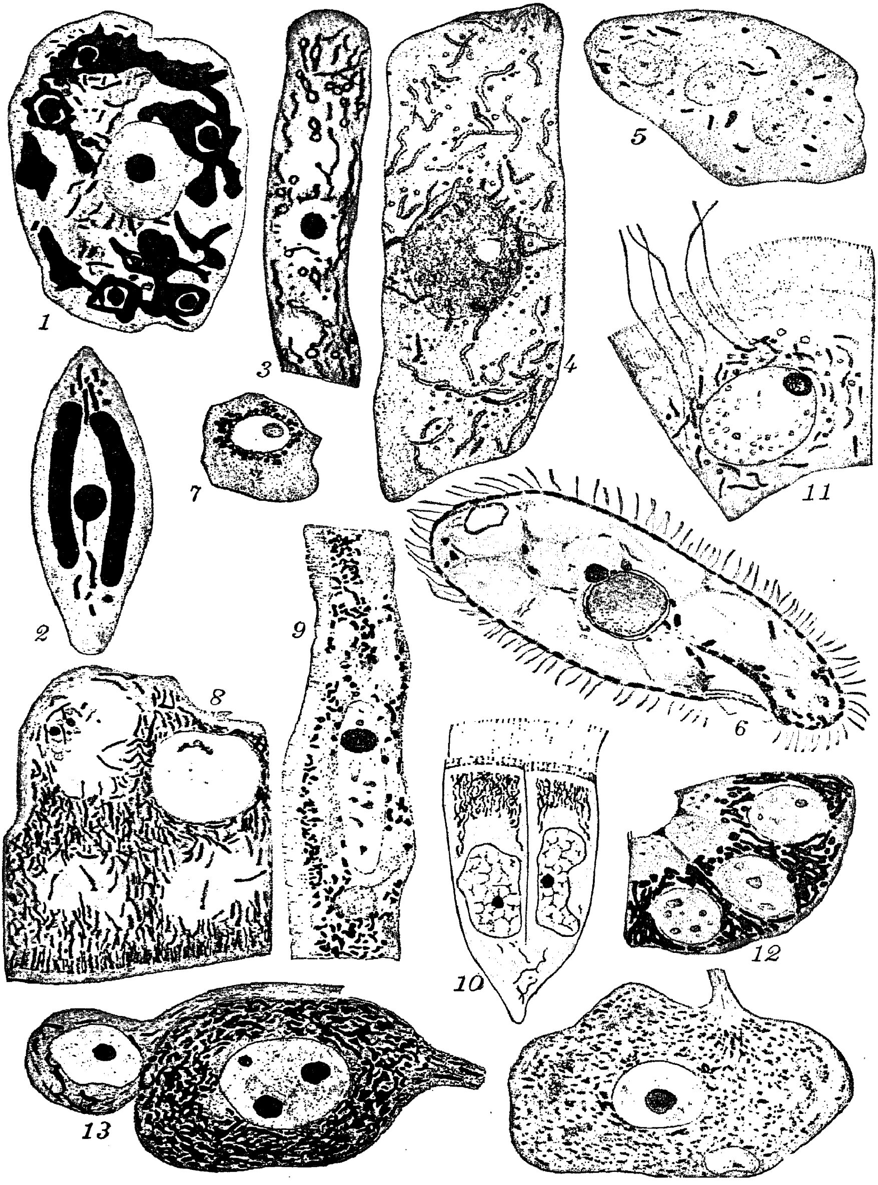
Very few animals or plants exhibit mitochondria of distinctive morphology, but if we compare the individual tissues of higher organisms we find considerable differences (see Figs. 1–14, p. 316). In some, filaments predominate, in others, granules of different sizes, but in similar tissues of different animals they are much the same. For instance, even in the different classes of vertebrates it is by no means a simple matter to distinguish the spinal ganglion cells on the basis of the morphology of their contained mitochondria (Cowdry, 1914*a*). The cells of the liver, pancreas, lungs, and other organs possess mitochondria which are alike in nearly related animals. This constancy in shape where the function is similar indicates that the morphology of mitochondria is a fundamental property ingrained in the organization of the cell, and that it is not always a passing, trivial affair which varies from moment to moment.

Mitochondria are often filamentous, particularly in gland cells (Fig. 11), in nerve cells (Fig. 13), and in most of the tissues of the developing embryos of all vertebrates. The average length of the filaments varies in different cases: They are very elongated in secreting cells like the pancreas, where they may attain a length of 10 to 12 microns (Fig. 18, p. 328). Their diameter also varies in different localities, but in individual cells of the same tissue it is astonishingly uniform. Filamentous mitochondria may be straight, curved, or even twisted, depending upon their surroundings. They do not taper toward their extremities but possess rounded ends. The uniformity in diameter must mean that interactions between the cytoplasm and the mitochondria can only profitably take place in a certain thickness of mitochondrial substance. We have therefore two attributes—length and breadth—independently variable and probably influenced by different factors.

When individual mitochondria under normal conditions increase in size, they probably do so through the addition of material at their extremities. The accretion is lengthwise, never lateral. It is equally true that when foreign material, like starch, pigment, or fat is deposited within the mitochondria, expansion is always provided for by increase in girth. No explanation is available of the difference in the mode of addition. Neither can we tell why two methods are employed for increasing the surface of mitochondrial material: that is to say, by elongation into filaments, and by frequent segmentation into rods and spherules of approximately the same diameter, so that a cell may be packed with filaments or with rodlike or spherical mitochondria.

It has been suggested that in some cases filamentous mitochondria may result from streaming movements in the cytoplasm as, for instance, in outgrowing nerve fibers and in gland cells. But they may be equally filamentous in bone cells and cartilage cells in which the cytoplasm is relatively stationary. Rubaschkin's (1910) idea that filamentous mitochondria are characteristic of specialized cells and granular forms of embryonic, undifferentiated cells is only of academic interest. Dubreuil's (1913) belief that filamentous mitochondria are indicative of rest and granular mitochondria of rapid multiplication by division has not been entirely substantiated by recent work; because, according to the descriptions of Moreau (1914*c*), they are granular in the spores of fungi in which cell activities are probably at a very low ebb; and, conversely, it has been found by N. H. Cowdry that they are filamentous in the rather inactive cells of the dried seed pea.

Occasionally mitochondrial filaments branch, and apparently give rise to more or less extensive networks. These are of comparatively rare occurrence, but are nevertheless normally found in certain types of cells, usually of secretory nature. The nets are often, but not always, arranged about the nucleus. The strands of the networks are generally of the same girth as isolated filaments in the same cells, except sometimes at their nodal points where they may be thickened.



We have abundant evidence that mitochondria are of semifluid consistency. In the streaming protoplasm of plant cells they are pliable and continually change in shape in response to currents and eddies in the stream. This semifluidity, in conjunction with their lipoid properties, precludes rough excrescences, sharp angles, and pointed ends. Variations in osmotic pressure (W. H. and M. R. Lewis, 1915) and changes in H-ion concentration (Cowdry, 1918) may be involved in molding their morphology, but there is no good reason to infer that these are the controlling factors (see p. 329). Although their causation remains a mystery, changes in the shape of mitochondria do constitute by far the most delicate criterion of many types of cell injury at our disposal. They tend to follow, moreover, in a definite sequence. First we often observe a breaking-up of filaments into granules (this may also be induced by faulty technique), then either a disappearance of the granules or their enlargement into coarse spherules. Coincident with this increase in size, the granules often blacken more readily with osmic acid, indicating perhaps an increase in their fatty acid content (see p. 325), which may be responsible for the calling into operation of the law of least surfaces.

5. *Occurrence:*

Comparatively few species have been examined in comparison with the magnitude of the phylogenetic series, but mitochondria have been found to occur in representative organisms ranging from man to the Protozoa and from the angiosperms to the fungi and Myxomycetes (Figs. 1–14). They have also been observed in certain algae and in diatoms (Guilliermond, 1921*e*) but their existence is doubtful in bacteria. Now that the first flush and excitement of discovery are past we may expect to have lowly plants and animals reported in which mitochondria do not occur or in which the materials said to represent them differ so widely from the typical mitochondria of vertebrates that the term cannot rightly be applied to them. For example, among the Protozoa, the piroplasmas pass through a stage of development within the red blood

FIGS. 1–14: FIG. 1.—Filament of *Spirogyra maxima*, after Guilliermond (1921*j*) containing typical rodlike and filamentous mitochondria. FIG. 2.—A diatom, after Guilliermond (1921*j*) containing similar mitochondria. FIG. 3.—A fungus, *Pustularia vesiculosa*, after Guilliermond (1915*b*). FIG. 4.—A spermatophyte, *Narcissus poeticus*, after Guilliermond (1919*c*). FIG. 5.—A myxomycete, *Arcyria denudata*, after N. H. Cowdry (1918). FIG. 6.—A protozoön, *Glaucoma piriformis*, after Fauré-Fremiet (1909). FIG. 7.—A coelenterate, *Aurelia aurita*, ovarian egg after Tsukaguchi (1914). Note perinuclear accumulation of mitochondria. FIG. 8.—An arachnid, *Amblyomma americana*, Malpighian tubule. FIG. 9.—An insect, *Cimex lectularius*, intestinal epithelium. FIG. 10.—An amphibian, *Rana esculenta*, pharyngeal epithelial cells after Saguchi (1917), showing distal condensation. FIG. 11.—A selachian, *Scyllium canicula*, cell from choroid plexus after Grynfeltt and Euzière (1913*a*), illustrating perinuclear clumping of mitochondria. FIG. 12.—Kidney cells of a white mouse with mitochondria in proximal cytoplasm. FIG. 13.—Small cell of *locus coeruleus* and large cell of mesencephalic nucleus of the fifth nerve of a white mouse to indicate difference in amount of mitochondria. FIG. 14.—Human spinal ganglion cell.

corpuscles of vertebrates in which the cytoplasm is very much reduced,[1] and *Anaplasma marginale* is said during this phase to consist wholly of nuclear material.[2] It would be a nice problem to ascertain whether with increase of cytoplasm mitochondria appear, because, if so, it would be a clear case of their *de novo* origin (see p. 323).

In the tissues of higher forms, mitochondria have thus far been found without exception, when adequate methods of technique have been used, except in cells whose vital activities are greatly reduced owing to the approach of senility. Instances like non-nucleated red blood cells will be mentioned subsequently (p. 321). There is evidence that the composition of mitochondria varies slightly in different cell categories as well as in different organisms.

6. *Arrangement within the cell:*

Within individual cells mitochondria are usually distributed without definite order throughout the cytoplasm, but some interesting exceptions are to be noted. For example, in the kidney the mitochondria are often most numerous in the basal region next to the blood vessels (Fig. 12). A similar arrangement obtains in all other glands with fixed polarity in which the direction of secretion is proximo-distal, that is to say, from the blood stream to the lumen of the duct. Champy has found that mitochondria are arranged differently in epithelial cells of the intestine where they tend to accumulate at both poles of the cell. This he believes to indicate the existence of a double polarization in two directions, for secretion and for absorption. It is interesting to note that Bensley (1916) is of the opinion that the original proximo-distal polarity of thyroid cells has been reversed and points to the heaping-up of mitochondria next to the lumen instead of near the peripheral blood vessels as one indication of a change in the direction of secretion. It is therefore possible that the mitochondria may serve to some extent as indicators of secretory polarity, like the Golgi apparatus (p. 336).

Perinuclear condensations of mitochondria occur in both plants and animals. For example, in the early meristem mitochondria are usually found indifferently distributed in the cytoplasm (Fig. 15). In older cells they seem to approach and come into actual contact with the nucleus, in which position they enlarge to form plasts (*B*) which then migrate away from the nucleus and finally become more or less evenly distributed in the surrounding cytoplasm (*C*). Guilliermond has repeatedly described this migration, and has found that the mitochondria undergo a parallel increase in resistance to the solvent action of acetic acid. Similarly, in the spermatogonia of certain animals the mitochondria make their way to the nucleus, and become so closely applied to it that some investigators have been led to think that they actually originate from it. In the later stages of spermatogenesis they leave the nucleus becoming more resistant to acetic acid, as Regaud (1910) has shown. They frequently also group themselves about the nucleus in ovarian eggs (Fig. 7). The Lewises (1915) have observed mitochondria journeying to the nucleus and back again in the living cells of tissue cultures. Here their movements are very rapid, and no change in composition is evident. Perinuclear condensations are of rather frequent occurrence in pathological conditions (see Fig. 26, p. 328).

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[1] H. Velu, 1923. *Les piroplasmes et les piroplasmoses.* Paris: E. Larose. 285 pp.

[2] Sir Arnold Theiler, 1910. "Texasfieber, Rotwasser und Gallenkrankheit der Rinder," *Zeitschr. f. Infektionskrankheiten der Haustiere,* **8**, 39.

Mitochondria are also prone to gather in the peripheral cytoplasm, especially in animal cells. This arrangement is very pronounced in eggs, and has often been alluded to by Van der Stricht and his pupils. After a time the mitochondria become redistributed, just as in the case of the perinuclear condensations. Grynfeltt and Lafont (1921*d*) have found that a peripheral margination of mitochondria may be induced experimentally in liver cells by sulfonal poisoning (Figs. 23 and 24, p. 328). Both perinuclear and peripheral condensations may normally occur synchronously, as in the ascidian eggs described by Loyez (1909).

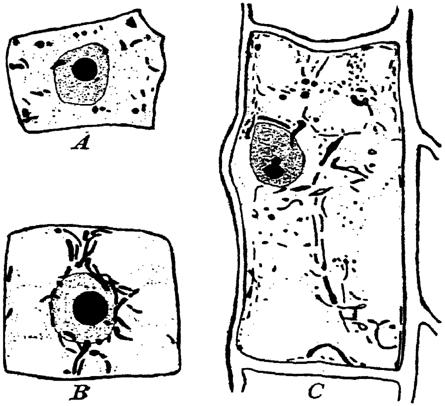

FIG. 15.—Meristem and young and old cortical cells of the pea showing: (*A*) primary diffuse arrangement of mitochondria; (*B*) secondary condensation about the nucleus, and (*C*) final dispersal throughout the cytoplasm (after N. H. Cowdry, 1917).

Mitochondria are often heaped up in the distal cytoplasm of ciliated cells (Fig. 10), but they are not always so (Fig. 11). They are also frequently clumped radially about the centrosomes. Other minor variations in their arrangement might be cited dependent upon the deposition of substances in the cytoplasm, upon pressure and other obvious causes. In these movements not a shred of evidence can be found that mitochondria possess powers of independent motility like some bacteria. Neither can they be fully explained by hypothecating mass movements of the cytoplasm. Kingsbury (1912) has suggested that the grouping of mitochondria about the centrosome, which is so often met with, may perhaps be interpreted on the supposition that, being reducing substances, they carry a positive electrical charge and accumulate around the centrosome in order to deliver it. It is also possible that the presence of an axial gradient in metabolism, as described by Child (1915), may be in part responsible for their distribution, particularly in gland cells. The Gibbs-Thompson principle tells us that any process which diminishes free energy at an interface will tend to take place. We have reason to suppose that mitochondria are lecithin-like, and we know that lipoids decrease surface tension, so that one would naturally expect them to be heaped up at the nuclear and plasma membranes. Perhaps we may be dealing with a manifestation of so-called electrical adsorption. On the other hand, the mitochondria may not be the directive agents. They may be shifted from place to place, like so many bricks, by physicochemical changes in the ground substance of which our best microscopes fail to give us any inkling.

7. *Amount of mitochondria:*

What is true in the case of morphology holds also here. In human tissues in which there is considerable division of labor we find many differences in the amount of mitochondria because some cells are best fitted to perform their duties with a large number and others with but few. From general information —we have no specific measurements—we cannot say that the cytoplasm of higher animals differs from that of those lower down in the scale in the amount of contained mitochondria. Neither is there any noticeable difference in the relative amount of mitochondria between animals and plants (compare the figures on p. 321).

In very young embryos of vertebrates most of the cells contain approximately the same amount of mitochondria. As development proceeds, tissues become specialized, and distinctive differences in the amount of mitochondria often become apparent. They are sometimes rather more abundant soon after birth than in adults.

Striking variations in the amount of mitochondria have been recorded in mature cells of different kinds. To estimate them quantitatively is particularly difficult in gland cells on account of cyclical changes in volume. It is necessary to secure a uniform degree of differentiation of the stain used. A beginning has been made in nerve cells in which the volume factor is not so disturbing. Thurlow (1917) has worked out a method of counting mitochondria by inserting in the ocular a glass disk with a ruled square of known dimensions and by using sections of known thickness. She discovered that there is a fairly constant number of mitochondria per unit volume of cytoplasm in the cranial nerve cells of white mice, so that certain groups of cells can be distinguished by the amount of mitochondria within them. This does not depend primarily upon whether the cells are sensory or motor.

In cellular senescence the amount of mitochondria is reduced. The reduction is proportionate to the formation of chloroplastids in plants (Fig. 16). It is said that when the plasts are fully formed, few if any mitochondria remain (Guilliermond, 1912*a*). In animals there is a similar disappearance of mitochondria in the life-cycle of red blood cells. In young, nucleated cells they are abundant but they are lost as the cell differentiates and dies (Fig. 17). In plants this disappearance is associated with the production of chlorophyll, in animals with the formation of hemoglobin—two substances having certain properties in common.

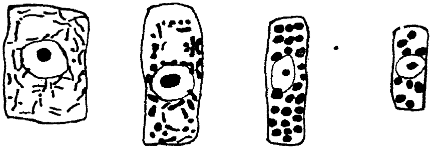

FIG. 16.—Meristem and parenchyma cells of the bean from N. H. Cowdry (1917) after Guilliermond (1912a), illustrating progressive disappearance of mitochondria with the formation of plastids containing chlorophyll.

We have two lines of observation to harmonize: this association of abundant mitochondria with intense protoplasmic activitv and a reciprocal relationship which appears to exist between the amount of mitochondria and the amount of fat (p. 329). Where there are few mitochondria there is often much fat, and vice versa. Decreased oxidation favors the deposition of fat and increased oxidation hastens its elimination, which suggests at once the existence of some connection between the amount of mitochondria and the rate of oxidation; and their abundance in the more active stages of the life of the cell, when protoplasmic respiration is rapid, points to the same tentative conclusion (see, however, pp. 325, 330).

8. *Composition:*

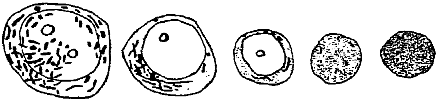

FIG. 17.—Differentiating red blood cells from the bone marrow of a rabbit supravitally stained with Janus green showing parallelism between the disappearance of mitochondria and the formation of a diffuse deposit of hemoglobin (after N. H. Cowdry, 1917).

The idea that mitochondria are chiefly of phospholipin nature with perhaps a small amount of protein in combination has never been actively questioned, but we naturally wish for a little more precise evidence than that which is at present available. The suggestion has come from three chief sources: from Regaud's (1908) study of mammalian tissues, from Fauré-Fremiet's (1910) work upon Protozoa, and from the investigations of the botanist Löwschin (1913, 1914), which latter have unfortunately never been confirmed. The evidence is largely negative, and may be found in detail elsewhere (Cowdry, 1918). Briefly, it consists of the solubility of mitochondria in alcohol, ether, chloroform, and similar reagents unless they have been rendered relatively insoluble by chromatization. The fact that their smooth and even outlines are suggestive of "myelin bodies"; the observation that they occasionally blacken with osmic acid and are related in some way to oscillations in the amount of neutral fat (p. 329) may be significant. Millon's reagent has been extensively employed in studies upon liver cells by Berg (1920, 1922), Noël

(1923), and others without obtaining a pronounced reaction on the part of the mitochondria. The mitochondria do not color with Sudan III or exhibit any of the properties of polysaccharides. Neither do they contain any appreciable amounts of iron in protein combination (Macallum's test). Observations tending to show that they are soluble at a temperature of 48° C. to 50° C. require confirmation. Since they act as solutes for various substances, they are often pigmented in plants, and assume quite brilliant hues. Among animals, the impregnation of mitochondria with yellow pigment has been noted by Gatenby (1919*b*) in *Limnaea*.

Although the reactions of mitochondria are usually uniform in cells of the same type, when we pass to other categories pronounced differences are apparent. The first detailed information has been supplied by Regaud (1910), who carefully compared the solubility of mitochondria in the cells of the testicle with respect to acetic acid. He found a progressive increase in resistance to acetic acid passing from spermatogonia to spermatozoa. Some years later N. C. Nicholson (1916) applied the same methods of technique to the central nervous system, and discovered that the mitochondria in certain cell types also differ in their solubility in acetic acid. It is a common experience to find that some experimentation is necessary before mitochondrial methods can be made to give wholly satisfactory results with unfamiliar tissues. Especially is this true in embryos as compared with adults and between specimens widely removed from one another in a phylogenetic sense. While these differences may be due to variations in the cytoplasm in which the mitochondria are imbedded, the possibility remains that the mitochondria themselves differ considerably in composition. Our present methods reveal their general properties, and perhaps gloss over differences which later on we may be able to detect. The physical consistency of mitochondria is discussed by Chambers on page 275.

9. *Function:*

Altmann (1890), believed mitochondria to be elementary micro-organisms imbedded in a lifeless ground substance. So striking is, in some cases, their morphological resemblance to bacteria that even to this day pathologists who see them for the first time usually exhibit a strong inclination to interpret them as independent micro-organisms. Since the time of Altmann, the idea that they are in truth bacteria which have become very completely adapted to an intracellular existence has been repeatedly advanced, particularly in recent years. A whole book and several pamphlets have been written in support of this contention by Portier (1917, 1918, 1919), but his view has not made a strong appeal to many investigators familiar with mitochondria among whom Regaud (1919), Laguesse (1919), Guilliermond (1919*a*), Rasmussen (1919), Van Gehuchten (1921), Caullery (1922), and Levi (1922) may be mentioned. In the United States, Wallin (1922, 1923*a–d*), quite independently of Portier, has advanced a similar hypothesis which has likewise been questioned by Cowdry and Olitsky (1922), and by Cowdry (1923*a*). The relation of intracellular "bacteroids" and of *Rickettsia* to mitochondria is discussed by Trojan (1919) and Cowdry (1923*b*) respectively. See also an editorial published in the *Journal of the American Medical Association.*[1]

The salient point in the discussion is the manner of inteerprtation of the differences which undoubtedly exist between mitochondria and bacteria. Wallin is of the opinion "that mitochondria are symbiotic bacteria in the cytoplasm of all higher organisms whose symbiotic existence had its inception at the dawn of phylogenetic evolution." It is natural that during millions of years their properties would become somewhat changed from free, living bacteria, and Wallin is inclined to explain some of the differences on this basis. Whether all differences are of a kind which might reasonably be expected to arise in this way may be seriously questioned. Neither can we accept without further and more detailed evidence his contention that other organisms which have enjoyed a symbiotic relationship for a relatively short time approach mitochondria in their properties to a degree which would lend any support to his theory. This he has claimed for the *Bacillus radicicola* and the organism of Rocky Mountain spotted fever as described by Wolbach, but very recently other investigators have clearly differentiated between these organisms and mitochondria lying side by side in the same cell (Cowdry, 1923*a*, and Nicholson, 1923*a*). Not only is there a very great gap between the properties of organisms which have developed the most perfect degree of symbiosis known to us and mitochondria, but the positive data which has accumulated regarding the latter does not readily lend itself to interpretation in terms of this hypothesis.

The idea which has dominated most of the work on mitochondria—that they are concerned in histogenesis—is not difficult to trace. They occur in all embryonic cells. In early stages of development they are the only formed elements in the cytoplasm. The conception that they are transformed into products of differentiation (Meves) falls also in line with the view that they are in part the material basis of heredity. The data bearing upon this theory has recently been summarized by Meves (1918*a*, 1918*d*). It is largely based upon the subsidiary hypothesis of mitochondrial continuity according to which mitochondria do not arise in the cytoplasm *de novo*, but always through multiplication by division of pre-existing units (see p. 318). While it cannot be denied from the evidence of tissue cultures (M. R. Lewis and W. H. Lewis, 1915) that in living cells some filamentous mitochondria do segment and break up into granules, it is very difficult to prove that all mitochondria arise exclusively from other mitochondria. A crucial experiment would involve the observation of living cytoplasm free from mitochondria, a condition rarely if ever met with. Mitochondria are described in spermatocytes on page 274, and their rôle in fertilization is discussed on page 470. For an account of their behavior in oögenesis, reference should be made to Van der Stricht (1923). The subject of cytoplasmic inheritance is considered on pages 600 and 725. A list of eighty substances in the formation of which mitochondria are said to be concerned was published by Cowdry in 1918. Many others may now be added. They comprise materials of the most diverse character, including glandular secretions, pigments, leukocytic granules, plant plastids, fibrillar structures of different kinds, fat, protein, glycogen, urea, etc.

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[1] *Op. cit.*, 79, 1848, 1922.

Unfortunately, the term "transformation" has been used too loosely without reflection as to what it means. Obviously, the likelihood of a transformation taking place depends upon the difference in the properties of the original substance and the supposed end product. Measured by this standard, some of the claims involve chemical and physical impossibilities. We have some reason to suppose that mitochondria are made up of phospholipins in protein combination. That masses of this material should bodily transform into hemoglobin which contains iron, chlorophyll which contains magnesium, and the colloid of the thyroid gland with its iodine is unlikely. These three elements are probably derived from the cytoplasm. The idea of transformation is often based upon the observation of different substances within the mitochondria, but this does not mean that they are of endogenous origin. That substances actually enter the mitochondria from without is often indicated by a distinct increase in size. For instance, mitochondria possessing bleblike swellings in gland cells are larger than those without them, and mitochondria containing starch, fat, pigment, and other materials are invariably greatly swollen. In cases where granular mitochondria expand to form vesicles, close observation will often show that there has been little or no change in the absolute amount of mitochondrial substance; it has simply been spread over a wider area, but there are other instances of a reduction in mitochondrial substance. We may safely regard imbibition from the cytoplasm as established, but how the substances are actually taken in it is difficult to explain. It differs from the normal process of growth because the expansion is, as we have noted, lateral; whereas in the growth of mitochondria, extension is usually longitudinal.

A more plausible eclectosome theory has been suggested by Regaud (1909), according to which the mitochondria are said to play the part of plasts choosing and selecting substances from the surrounding cytoplasm, condensing them and transforming them in their interior into infinitely diverse products. He has compared the mitochondria to the hypothetical side chains of Ehrlich. His theory is essentially a modification of the lipoid membrane conception of Overton, the difference being that the lipoid is considered to be scattered throughout the cytoplasmic area, in the form of mitochondria, instead of being confined to a layer on the surface of the cell.

But it is not necessary to endow the mitochondria with any wonderful anthropomorphic attributes, for they may equally well act in a more or less passive way, taking up materials by virtue of their peculiar constitution, or on account of physical forces acting on their surfaces. We can conceive of chemical changes arising: (1) by the addition of substances from the cytoplasm which enter into close combination and become integral constituents of the mitochondria; (2) by the mitochondria giving up to the cytoplasm certain of their normal constituents; and (3) by chemical dissociation and by chemical resynthesis within the mitochondria.

However this may be, the recognition of a definite topographic relationship between mitochondria and many products of cellular activity is a very great advance in our knowledge of vital processes, comparable even with the discovery of chromosomes. But this is not the whole story. Mitochondria are almost, though not quite, coextensive with vital phenomena. In the cells of all embryos they take part in vital activities of a generalized and fundamental type before the onset of specialization. Kingsbury (1912) was the first to suggest that they function in protoplasmic respiration. He says that osmic acid, potassium bichromate, and formalin are the chief ingredients of mitochondrial fixatives and that their value depends upon the presence of reducing substances in the cytoplasm. These he believes to be the mitochondria on account of their lipoidal properties. More detailed evidence is given by Mayer, Rathery, and Schaeffer (1914*b*). These investigators contend (1) that mitochondria are phosphatids containing unsaturated fatty acids with ethylidene groups, and are therefore chemically well adapted to function in oxidations and reductions, and (2) that agents which attack lipoids (like alcohol, ether, and chloroform among anaesthetics) at the same time cut down respiratory oxidations. It is interesting also to note that what we know of the Janus green reaction (p. 313) seems to lead to a similar conclusion. But although this view, that mitochondria take part in protoplasmic respiration, has been well received by cytologists and serves as a useful and convenient working hypothesis, it is still only a theory and must be regarded as such.

10. *Changes in pathological conditions:*

Unfortunately observations in this field are often disguised by the use of misleading terms to designate the mitochondria (see p. 313). Some confusion has also arisen from the fact that we have to do with two mitochondrial literatures—an old and a new. The first developed as an outgrowth of Altmann's remarkable researches at Leipzig from 1880 to 1890. It flourished for a time, and became much more extensive than is generally supposed, but it was soon submerged by the active criticism which his views excited. The second, in which we now find ourselves, is an expression of a revival of interest in the cytoplasm, and is coincident with a tendency among physiological chemists and pathologists to become interested in the phospholipins, whereas formerly their whole attention was devoted to the study of proteins, and was dominated by the tremendous impetus of Emil Fisher's studies on protein synthesis based upon Kossel's theory of the nature of the protein molecule. A potent psychologic factor was thus involved in the idea of making living substance. It is not unlikely that contemporaneous studies in heredity also tended to focus attention upon the nucleus. Investigators very naturally chose fixatives which would reveal nuclear detail, forgetful of the destruction which they sometimes wrought in the cytoplasm. The classical mixture of Zenker is a case in point. The 5 per cent of acetic acid which it contains helps to give a uniform preservation with sharp, nuclear detail, but its solvent action is usually sufficient to remove all traces of the mitochondria normally present within the cells; similarly with certain alcoholic fluids, so that the technique in vogue in some measure excluded and certainly retarded the recognition of mitochondria. A kind of vicious cycle developed.

With the elaboration of more satisfactory methods for the study of mitochondria, it became at once evident that we have at hand a relatively new criterion of cell activity and of cell injury. Since the mitochondria differ radically from the nucleus, the belief has been frequently expressed that their study will open an entirely new chapter in cellular pathology. It is true that a systematic and comprehensive investigation of the behavior of these cytoplasmic indicators may confidently be expected to yield information of transcendent importance which will supplement and extend the older work based almost wholly upon the observation of nuclei. The cytoplasm, being perhaps more directly concerned with adjustments between the living cell and its environment, will in all likelihood be very responsive to harmful influences. The possibilities involved are indeed alluring.[1]

Animated by the belief that this line of advance might prove to be a short cut to a better understanding of the nature of pathological processes, mitochondria have been rather hastily studied in a great variety of conditions. Some of the papers have already been summarized (Cowdry, 1918), and the bibliography at the end of this section contains references to many others, so that at this stage it requires some knowledge and no little imagination to select any type of injury in which the mitochondrial changes have not already been touched upon. Unhappily, attempts have usually been made to strike right at the root of the problems involved by the examination of human tissues taken either at operation or at autopsy in which adequate control is out of the question because the conditions can never be exactly duplicated. Failure to recognize the fact that an unusually delicate criterion of cellular injury of this kind, like a new and little-known chemical reaction, is likely to be misleading, is responsible for some disappointment in the results obtained. It cannot be overemphasized that however precise the instructions may be, the technique cannot properly be shifted on to shoulders of a technician. The methods must be adapted to each individual tissue, and must be standardized by repetition again and again. Even a slight and apparently insignificant deviation from the routine will often produce unexpected and profound alterations in the mitochondria. Pinching the tissues slightly with the forceps, or letting their surfaces dry in air will render them useless. Much has been written about the advisability of taking tissues very soon after death. This depends, of course upon the rate of autolysis. It is particularly necessary with glands and less so with the nervous system.

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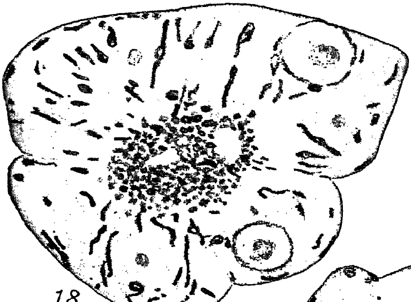
[1] See editorial, *J. Am. M. Assoc.*, 67, 813, 1917.

For many years, therefore, the study of mitochondria in pathology will involve the painstaking observation of their behavior in animals under experimental conditions which are capable of rigid control, despite the fact that the spice of novelty has been in most cases removed. A good beginning has already been made in this direction. For instance, it has been shown that whereas the mitochondria are wonderfully sensitive to any interference with the normal activity of some tissues, they are unexpectedly resistant in others. In glands they often respond by a loss of filamentous shape a considerable time before the nuclei exhibit noticeable modifications. This has been clearly shown to result in the pancreas from phosphorus poisoning, by Scott (1916). Some of his drawings are reproduced in Figures 18–26 (p. 328). It will be seen, as compared with the normal (Fig. 18), that the mitochondria first lose their filamentous shape (Fig. 19), then clump together and agglutinate (Figs. 20 and 21), and finally fuse, giving rise to the familiar droplets of fatty degeneration (Fig. 22). By systematic experimentation, Nicholson (1923*b*) has brought to light a variety of mitochondrial changes in the thyroid which would probably have been completely overlooked in ordinary preparations fixed in Zenker's fluid and stained with hematoxylin and eosin, because the nuclei are only slightly altered (see Fig. 27). Other instances of the extreme susceptibility of the mitochondria in gland cells might be cited from the almost overwhelming literature. It is quite otherwise in the nervous system, although the mitochondria do respond to serious injury like axone section (Luna, 1913*c*). Thus, Clark (1914) was unable to detect conspicuous changes in experimental beri-beri, Strongman (1917) in functional exhaustion, or Rasmussen (1919) in hibernation, and McCann (1918) found normal filamentous mitochondria in nerve cells in fairly advanced stages of chromatolysis in poliomyelitis, while in experimental herpetic infections of rabbits, Cowdry and Nicholson (1923) observed apparently unaltered mitochondria in nerve cells greatly plasmolyzed and exhibiting profound nucleolar alterations. No explanation has yet been advanced to explain this variability in the reactivity of mitochondria to cellular injury, but it may be a function of the properties of the cytoplasm in which they are imbedded, and may not signify any radical difference in the mitochondria themselves.

Three general modes of reaction are recognized—qualitative, quantitative, and topographical, which may occur singly or in combination.

By far the most delicate qualitative response is a change of filamentous mitochondria into granules, but the exact method by which it is produced is



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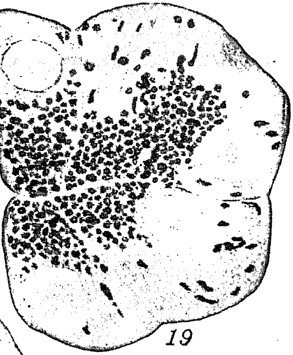

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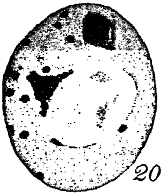

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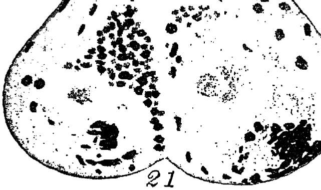

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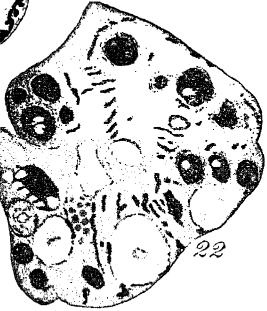

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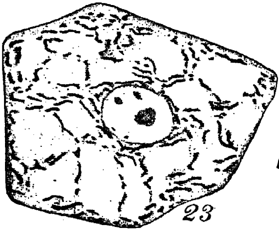

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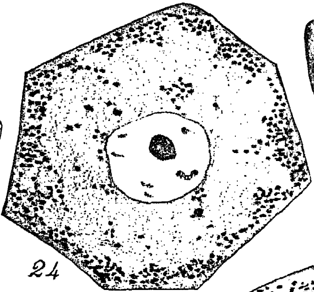

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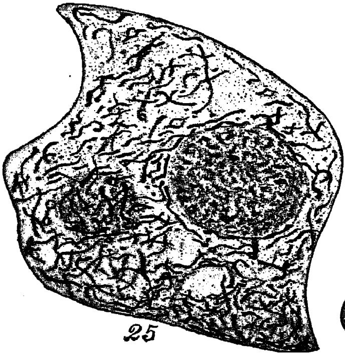

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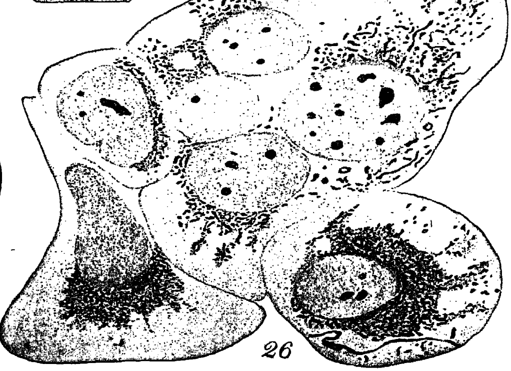

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not definitely known. It would appear that in most cases the filaments break up into segments which round up into spherules, but it is possible that individual filaments may give rise to single droplets. Attention has already been called to the fact that this granulation of mitochondria is often merely a manifestation of faulty technique. It may be provoked by many kinds of experimental injury, such as phosphorous poisoning (Scott, 1916; Nicholson, 1923*b*), sulphonal poisoning (Grynfeltt and Lafont, 1921*a–e*), inanition (Miller, 1922; Okuneff, 1923; Ma, 1923), and has been observed in the living cells of tissue cultures (W. H. Lewis and M. R. Lewis; Levi). Evidently, therefore, cytoplasmic conditions, favorable to a filamentous form of mitochondrial material, may be disturbed in different ways, so that the reaction cannot be regarded as in any sense specific. We cannot tell whether the poison, or other injurious influence, acts more or less directly upon the mitochondria or whether the alteration in the mitchondria is only the visible expression of a long line of interdependent chemical reactions. Occasionally an increase in the girth of mitochondrial filaments is noted but very rarely are they observed to elongate as a result of injury. Quite frequently they swell up into droplets with pronounced fatty and lipoidal properties. These morphological changes will remain very obscure until we are able to correlate them with chemical changes by the aid of more exact methods for the detection of the lipoidal and protein fractions which probably enter into the composition of mitochondria. Changes in the form of mitochondria produced in living cells outside the organism by the action of hypo- and hypertonic solutions, by acids and bases, and by alterations in temperature are interesting and significant, but the limited range of variation in these qualities of the circulating blood precludes their operation, except in a minor degree, and we are left to explain why mitochondria, existing side by side in the same cell, and sharing many influences in common, often differ so greatly in morphology.

Quantitative changes in the mitochondria are equally found to be misleading. Even with uniformly fixed tissues, unless the stain is differentiated to exactly the same extent, an illusory impression of decrease or increase in mitochondria may easily be created. With few exceptions, the observations recorded in the literature are based upon the general appearance of sections. But few investigators have availed themselves of the more logical method of actually counting

FIGS. 18–22.—The effect of phosphorus poisoning upon the mitochondria in the acinus cells of the guinea pig's pancreas (after Scott, 1916). From the normal condition we note a loss of the filamentous shape of the mitochondria with a rounding up into granules, an agglutination of the granules, and their fusion into droplets of fat.

FIGS. 23 and 24.—The rounding up of normal filamentous mitochondria in the liver cells of the rabbit (1923) into spherules and the migration of the spherules into the peripheral cytoplasm under the influence of sulphonal poisoning (after Grynfeltt and Lafont, 1921*e*).

FIGS. 25 and 26.—From a mammary gland carcinoma in a woman of forty-five years of age, to illustrate the variability in the number, shape, and arrangement of the mitochondria (after Favre and Regaud, 1911).

the mitochondria in unit areas (Thurlow, 1917; Rasmussen, 1919), and have taken the trouble to measure accurately the variation in the size of the cells (Noël, 1923). In practice, a diminution in the number of mitochondria is commonly met with in pathological conditions, but a definite increase above normal is rare. It has, however, been reported in compensatory hypertrophy (Enderlen, 1908; Hirsch, 1910; De Giacomo, 1911; Nicholson, 1923*b*, and others) and during regeneration (Romeis, 1913*c*; Torraca, 1914*a*, *b;* 1916). In toxic adenomata of the thyroid, mitochondria are also increased (Goetsch, 1916). In tumors great variability is noticeable (Veratti, 1909; Beckton, 1909; Bensley, 1910; G. Arnold, 1912*a;* Favre and Regaud, 1913*a*, *b;* Poricelli-Titone, 1914; and Sokoloff, 1922). The illustrations of Favre and Regaud, reproduced in Figures 25 and 26, will bear careful examination. The assumption is perhaps warranted that a decrease in the number of mitochondria is a sign of depression of functional activity, and that an increase is indicative of heightened activity, provided that they retain their normal shape but, when the increase is manifested by a swelling up of mitochondria into rounded droplets of different sizes, the condition is apt to pass insensibly into a simple accumulation of fat and lipoid which would point, on the other hand, to a

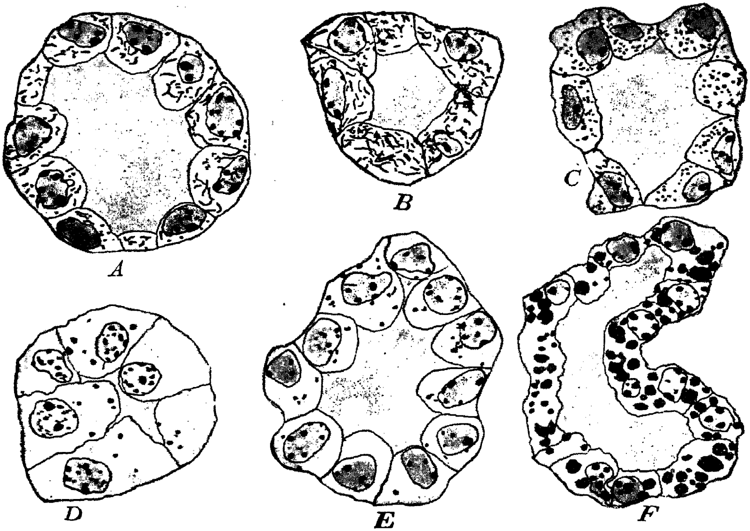

FIG. 27.—The reactions of mitochondria in the thyroid gland of guinea pigs: (*A*) the normal; (*B*) hypertrophy of remaining fragment after the removal of one gland and half the gland on the opposite side; (*C*) inhalation of oxygen; (*D*) ligation of blood vessels; (*E*) fasting; and (*F*) phosphorus poisoning (after Nicholson, 1923).

decrease in the rate of intracellular oxidation. For the present, even a conspicuous increase in filamentous and rodlike mitochondria, or of tiny granules, hardly justifies the conclusion that any specific type of physiological process is enhanced, but it does show that the cells are not in a dormant or inactive state.

By contrast, changes in the position of mitochondria within the cell are much less likely to result from imperfect technique. They have been reported in several conditions. The peripheral margination in sulphonal poisoning, discovered by Grynfeltt and Lafont (1921*a–e*), is particularly noteworthy (compare Figs. 23 and 24). A grouping of mitochondria about the nucleus is also of fairly common occurrence, and is generally, but not invariably, associated with alterations in form (Fig. 26). Here also interpretation is our stumbling-block, and will probably only give way to a systematic comparison on a large scale of experimental conditions which cause a movement of mitochondria to and from the nucleus, concerning the possible mechanism of which suggestions have already been made (pp. 319, 320).

But mitochondria are of value in cellular pathology, not only as indicators, but in other ways. Recognition of the fact that they occur in the vast majority of living cells opens up a new line of study in the re-examination of the genesis of cytoplasmic inclusions of doubtful nature, described before their discovery. For example, the large group of exanthematic diseases of unknown etiology comprising vaccinia, small-pox, scarlet fever, rabies, chicken pox, and several others that are less familiar offers many remarkable cytoplasmic inclusions concerning which further information is urgently needed. The remarks made on page 324 may here be helpful as a point of departure in the selection for examination of those bodies which may conceivably be of mitochondrial nature or derived from them.[1]

11. *Outlook for further study:*

Unfortunately, the positive achievements of the study of mitochondria are as yet somewhat intangible. They constitute more than anything else a good augury for the future. However, when finally we attempt to take stock of the situation, several points become clear to us.

In the first place, it is quite obvious that the investigation of mitochondria will never achieve the usefulness which it deserves as an instrument for advance in biology and medicine until we know much more of their chemical constitution as the only accurate basis for interpretation of our findings. In other words, we must wait upon the slow development of direct, qualitative cellular chemistry.

Secondly, we have to admit that we are still, more than thirty years after the discovery of mitochondria, working very much in the dark. It is not greatly to our credit that these elements, which we can all clearly see at will in living cells, merely by the addition of a drop of Janus green and the use of a good microscope, should continue to remain such a profound mystery. Neither is it

[1] For recent literature see: S. S. Wail, *Arch. Path. Anat.*, **256** (1925), 518.

gratifying to find how relatively insignificant has been our progress in the last five or six years. We have a plethora of observations but no new experimental method has brought us noticeably nearer to a solution of the puzzle. It is possible that we, as students of mitochondria, have allowed ourselves to become rather narrow, and have approached too closely to the problem to see it in its proper proportions, whereas our task is in reality a synthetic one: we must piece together information from many quarters, and build up in our mind's eye a dynamic picture of mitochondria in relation to innumerable other cellular constituents. To take a familiar example, the close study of the mainspring of a watch would not tell us very much unless its behavior was carefully considered in connection with all the other parts of the mechanism.

We must continually search for new methods of approach, and strive to use to better advantage methods already at hand. Even in experimental animals the physiologic processes are of such complexity as to be very baffling. To simplify matters we may have to resort to the systematic examination of pure lines of cells in tissue cultures by methods like those recently elaborated in a series of contributions by Carrel and Ebeling (1923) which make possible quantitative study and the accurate analysis of cellular behavior in media of known chemical constitution. We may also profit by the very considerable advances made by W. H. Lewis and M. R. Lewis, Levi, and other cytologists with saline solutions as media.

In the past the tendency has been to single out the mitochondria and to study closely their behavior under many conditions. The time may have come to attack the problem from a slightly different angle. It is a sign of the times that more and more emphasis is being placed upon the interdependence of vital processes. Thus, Osterhout (1922) is of the opinion "that life depends upon a series of reactions which normally proceed at rates which bear a definite relation to each other" and "that a disturbance of these rate relations may have a profound effect upon the organism, and may produce such diversive phenomena as stimulation, development, injury and death." Certainly the most wonderful attribute of a living cell is the harmony of the chemical processes taking place within it. May we not profitably shift our method of approach and strive to modify cellular activity as nearly as possible in one direction only and if possible to a degree that can be quantitatively measured, and then proceed to an intensive examination of the interrelationships of the resultant structural and chemical changes? Correlations might well be effected between the composition of the medium in which the cells are living, some physiological attribute which can be measured and reduced to mathematical terms like growth, the size and internal morphology of the cells, and with their microchemistry. The more skilful the analysis in selecting likely variables, the better would be our chance to unearth phenomena causally related, and thus to obtain an inkling of the method of integration of vital processes within the cell and of the part played therein by mitochondria.

In these days when attention is rightly focused upon physical forces acting at surfaces of separation between fluids of different character and density, the discovery, that one of the most fundamental features of cellular architecture is the presence of almost innumerable mitochondria which in the aggregate afford a surface far greater in extent than that of the nuclear and plasma membranes, is suggestive. While fibrils, secretion granules, centrosomes, and all other known products of protoplasmic differentiation are but short-lived, and even the nuclear membrane is (during cell division) periodically lost and reconstructed, the mitochondria-cytoplasmic complex, alone, is very nearly inseparable from phenomena which we call vital, and has so endured for years without number. The topographic association between mitochondria and chemical changes of great variety has already been alluded to. It is happily not a theory, but a fact established by direct and repeated observation. But as we only dimly appreciate the meaning in cellular physiology and pathology of the more familiar transitory and permanent membranes and surfaces of separation, so it may be many years before we even approximate to a correct interpretation of the mitochondria. (This subject is further discussed in its physical aspects on p. 177.)

II. GOLGI APPARATUS[1]

1. *Discovery:*

The Italian neurologist, Golgi (1898), was the first to observe the cytoplasmic component which bears his name in nerve cells after treatment by a slightly modified method of silver impregnation. He wisely expressed himself with great reserve regarding its nature. About the time that this discovery was announced, Holmgren (1899), Nelis, (1899), and several other investigators found a system of clear canals within the cytoplasm of a large variety of cells belonging to the same categories in which the Golgi apparatus was being found. These were seen after many fixatives, and exhibited the property of remaining uncolored when the rest of the cytoplasm was stained. Their close resemblance in form and position to the apparatus of Golgi attracted widespread attention, so much so that Cajal (1908) felt justified in proposing the name of Golgi-Holmgren canals to include both formations. This action has, however, not passed unchallenged (see p. 341). As in the case of mitochondria, it was some years before the morphologic independence of this new cytoplasmic structure and others described previously was proved. The early literature is best reviewed by von Bergen (1904).

2. *Definition:*

Progress has been very slow. Even now, twenty-five years after its discovery we can only say that the Golgi apparatus is an area of the cytoplasm frequently (especially in higher forms) of reticular shape, often as large as the nucleus, and sometimes definitely located in relation to cellular polarity. Part of the material of which it is composed is soluble in alcohol, becomes blackened after prolonged treatment with osmic acid, and, after appropriate preliminary fixation, shows a marked affinity for silver salts. In addition it may occasionally be stained with resorcin-fuchsin, iron hematoxylin, and other dyes, but the word "apparatus" is unfortunate because it carries with it the idea of a mechanism of rather mechanical type.

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[1] Synonyms: binnennetz, urano-argentophile apparatus, and about twenty-five other terms (Cowdry, 1921).

3. *Technique:*

The most up-to-date résumé of technique is given in the last edition of Lee's *Vade-mecum*, edited by Gatenby (1921). Methods have not yet been devised by which the Golgi apparatus may be studied in the living cells of vertebrates. We have to rely upon rather unsatisfactory osmium and silver preparations between which there is little to choose. Both require considerable experience in cytology and constant personal attention. It will not suffice merely to follow instructions, no matter how detailed they may be. Careful experimentation is required with each tissue, and great caution has to be exercised in interpreting the findings, particularly in respect to changes in the shape and size of the Golgi apparatus occurring normally and induced experimentally.

4. *Morphology:*

Usually in the somatic cells of mammals and the majority of vertebrates, the Golgi apparatus is seen in the form of a more or less dense network consisting of anastomosing strands of material of uneven girth but of smooth contours. The fact that it is never of exactly the same shape even in neighboring cells of the same kind suggests a high degree of plasticity and the probability that it is continually changing in form from moment to moment. It may be closely drawn together into a rather compact mass or dispersed throughout the cytoplasm in isolated fragments (as in some nerve cells). Variations in the thickness of the strands are often the result of faulty technique which sometimes also brings about a fragmentation of the apparatus into droplets of irregular size. But well-controlled preparations show conclusively that the morphology of the Golgi apparatus is in a general way typical in different cell types. For instance, its shape is quite different in the acinus cells of the pancreas and in polymorphonuclear leukocytes (Cowdry, 1921). And further, if the pancreas is examined in several different groups of animals, the same style of Golgi apparatus is encountered. In other words, variations in its morphology are closely related to variations in cellular organization and function.

In the lower Metazoa the information at hand appears to show that taken generally the Golgi apparatus shows a tendency to occur in isolated rods and spherules instead of complicated networks. Particularly is this true in stages of oögenesis and spermatogenesis. Isolated fragments are also met with in the

Protozoa. To this circumstance is due the practice of some writers in speaking of Golgi "bodies." The word "body" unavoidably carries the impression of distinctive form and to some extent of solidity, as contrasted with relative fluidity.

5. *Occurrence:*

In the Metazoa the Golgi apparatus is certainly of very wide occurrence, but Gatenby (1919*b*) may be a little premature in his statement that it or its representative is present in every cell of vertebrates and invertebrates. Non-nucleated red blood cells are the first exceptions to suggest themselves and others might be cited.

Since we seem to be dealing fundamentally with unknown substances in watery solution, or in a matrix which may be more dense than the rest of the cytoplasm, we cannot do more than merely outline their distribution, with due qualifications, and in the most tentative way. Borderline cases are perplexing because the material of which the Golgi apparatus is composed unquestionably varies progressively in the life-history of specialized cells so that it is difficult to say when we are dealing with a true Golgi apparatus or with other materials which may be in part its products. The same uncertainty is met with in the case of mitochondria. With our present technique we can just skim the surface and recognize a few of its most general attributes. When, after a long period of experimentation, we are at last in a position to delve more deeply into its properties with refined methods, we may confidently look for differences in cells of different categories. For the present, progress can well be made by establishing an elastic and very tentative type of homology through the collection of data regarding the morphology and biologic behavior of the apparatus, as advised by Hyman (1923).

A Golgi apparatus has been described in Protozoa first by Hirschler (1914) and later by King and Gatenby (1923). Thus far it has not been reported in bacteria, in algae, nor yet in fungi. The presence of structures probably very nearly related to it has been noted by Bensley (1910), Pensa (1910, 1917, etc.), Laburu (1916), Drew (1920), Guilliermond and Mangenot (1922), as well as several other investigators, in the young and immature cells of plants (see pp. 343, 344).

Turning now to individual tissues we find that some representative of a Golgi apparatus is to be seen in egg cells and in all the cells of developing embryos. On cell division the networks are broken up into smaller masses (resembling to some extent the Golgi "bodies" of lower forms) which are distributed approximately equally to the two daughter-cells, in which the networks are again reconstituted. Sometimes this process is characterized by great regularity; at other times it has the appearance of being rather haphazard. With growth and differentiation, they pass through a definite sequence of changes suggesting, as pointed out by Bensley, that we have to do with a material which behaves as a unit in the developmental cycle. If we search through adult tissues, we find the Golgi apparatus present in one form or another in each and every cell, except in those which are dead and dying, like the mature erythrocytes already mentioned and desquamating epithelial cells.

6. *Position in the cell:*

We owe to Cajal (1915) the observation that in the developing embryo the Golgi apparatus in the ectodermal cells is always present in the cytoplasm between the nucleus and the periphery. It even maintains this position when the ectoderm is reversed, as in the formation of the optic vesicles.

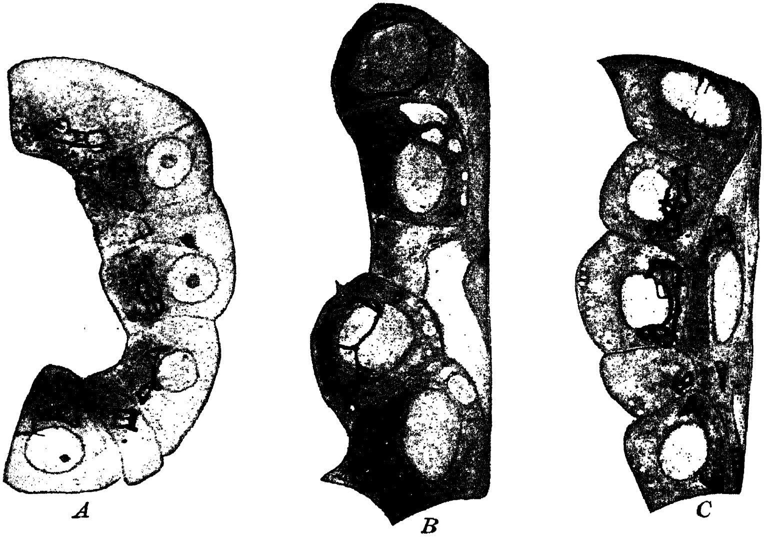

FIG. 28.—(*A*) Thyroid follicular cells of a normal adult guinea pig prepared by Cajal's uranium nitrate and silver method with the Golgi apparatus in its usual position between the nucleus and the lumen. (*B*) Showing migration of the Golgi apparatus toward the lumen. (*C*) Illustrating movement of the Golgi apparatus in the opposite direction away from the lumen and toward the periphery.

Another point of interest, and perhaps of immediate practical importance, is that the Golgi apparatus in gland cells appears to move about in the cytoplasm in a remarkable and orderly manner. In cases where the secretory polarity is fixed, as in the acinus cells of the pancreas, and the cells of the salivary glands, it seems to be always placed between the nucleus and the lumen. In the thyroid, on the other hand (Fig. 28), it has been found that at least in the adult guinea pig it normally migrates from one end of the cell to the other (Cowdry, 1922). That is to say, from its usual position between the nucleus and the follicular cavity (Fig. 28*A*), it may approach the lumen (*B*) or flow around the nucleus to the opposite pole of the cell adjacent to the peripheral vascular network (*C*). This reversal may take place in entire follicles or within single cells. There seems to be a kind of ebb and flow. In this respect the thyroid is perhaps comparable to the choroid plexus as studied by Biondi (1911). A somewhat similar but progressive change in the position of the Golgi apparatus has also been reported by Golgi (1909) in mucus-secreting intestinal epithelial cells.

Since we have reason to believe that the thyroid differs from other glands like the pancreas in being able, under conditions which are but little understood, to pour its secretion directly into the peripheral vascular network, the suggestion has been made (Cowdry, 1922) that we have in these migrations a real indicator of physiologic reversals in polarity. If this theory is confirmed, experimentally, preparations of this type, revealing the position of the Golgi apparatus, may afford accurate information regarding the direction of discharge at the moment the tissues are taken. In this way it may well be possible to effect a close correlation between the actual discharge of thyroid secretion and the response by the organism to its action. The likelihood of success with the thyroid is indicated by Masson's (1922) researches on the position of the centrosomes in malignant thyroid tumors. It has been abundantly shown that the centrosomes are closely related in a topographic sense to the Golgi apparatus. Like the Golgi apparatus they usually lie in thyroid cells between the nuclei and the lumen. But in tumor cells Masson found them to migrate in the direction of the peripheral blood vessels. In one of his illustrations it will be seen that though most of them are in the usual position, a few are reversed. In another, all are reversed, and the colloid has disappeared from the lumen, and has accumulated about the peripheral blood vessels. The follicles seem to have been completely turned inside out in respect to the direction of secretion. Successful preparations of the Golgi apparatus would probably have revealed the same phenomenon.

A study of the position of the Golgi apparatus in the parathyroids of new-born kittens has been made by Courrier and Reiss (1922). With the apparatus as an indicator, they claim to have established the existence of a definite secretory polarity and the presence of a network of capillaries of two types. They believe that at this stage the cells are arranged in cylindrical columns, the surfaces of which are bathed by nutritive capillaries and the central areas drained by others of excretory nature, and that the position of the Golgi apparatus between the nucleus and the central capillary indicates the existence of a definite functional polarity. The authors point out that this discovery of polarity gives us a somewhat new conception of endocrine cells, it having been customary thus far to deny the presence of definite secretory polarity in all of them except the thyroid.

Reiss (1922) has also investigated the anterior lobe of the cat's hypophysis in which it will be recalled that the three types of cells generally recognized appear to be related. From being chromophobe, they appear to become basophile and then acidophile. In the first named, the apparatus is, according to Reiss, without special orientation which corresponds nicely with the view that these cells are resting. In the basophile cells it is invariably found between the nucleus and the periphery of the cluster, and finally in the acidophiles it is located between the nucleus and the central area. Reiss claims to have observed all transitional stages in this sequence. His interpretation is that we have a mechanism by which the cells are able in one stage to pour a secretion toward the periphery and then to turn about face and discharge a second and different product into the center of the cluster. It is questionable whether these suggested oscillations in secretory polarity could have been detected without the clue offered by the migration of a conspicuous structure like the Golgi apparatus.

A significant observation to be emphasized is that reversal in the position of the Golgi apparatus may also be induced experimentally in tissues in which it does not occur normally. D'Agata (1910) discovered a change in position in epithelial cells of the newt's stomach following scarification, and Basile (1914) found that it could be reversed in the cells of the straight and convoluted tubules in one kidney through the extirpation of the other. From its normal position between the nucleus and the lumen it migrates to the opposite pole facing the peripheral blood vessels. Unhappily, these observations have not been confirmed, but the illustrations presented by Basile are so clear and convincing that it is difficult to imagine how he could have gone astray. But we must not hasten to the conclusion that the purpose of the Golgi apparatus is to elaborate secretion, because we find it to be equally highly developed in nerve cells and in others in which secretory activities are not pronounced. Neither can we say that in gland cells it is wholly unrelated to the formation of secretion, since so marvelous an integrating and unifying principle is manifest in all vital processes. We have to steer a middle course. What the observations which have been related do show is that we are now able to follow significant changes in the position of an important and hitherto unrecognized cytoplasmic area. From the technical point of view we are on firmer ground than in the study of variations in the shape and size of the Golgi apparatus, inasmuch as it is altogether unlikely that any error in manipulation would constantly bring about so definite a shifting in the relative position of parts of the cytoplasm.

7. *Size:*

The data to be considered are very scanty, and will remain so until much needed improvements are made in technique. Any quantitative study is beset with almost insurmountable difficulties. Slight variations of unknown origin constantly occur. It cannot even be said whether the Golgi apparatus is relatively larger in proportion to cell volume in the different classes of vertebrates, but some cells are evidently fitted to perform their duties with a large Golgi apparatus (gland cells) and others with a relatively small one (muscle cells); and further it is known that the Golgi apparatus is usually well developed in active stages of cytomorphosis, becoming progressively smaller as the cell ages (except in plants, p. 344), until it finally disappears with senility and death. There is also satisfactory evidence that this peculiar cytoplasmic area becomes definitely enlarged in certain pathological conditions (p. 348). For instance, Tello (1913) discovered a marked degree of hypertrophy in tumors of the mammary gland, and his results are in harmony with an increase in the size of the apparatus, which Da Fano (1922) subsequently noticed in the mammary gland during pregnancy and lactation. Other examples might be cited from the extensive literature, but we shall do well to err, if at all, upon the side of conservatism in recording experimental changes and to accept only alterations which are very pronounced, because slight variations in size occasionally occur spontaneously without apparent rhyme or reason. Some of them may be due to slips in technique or to the influence of changes in light or temperature upon the silver reaction or the blackening with osmium.

Passing now to a consideration of the actual mechanism of changes in size, we must at once plead complete ignorance. There has, however, been no dearth of speculation. Attempts have not been wanting to bring the "Golgi bodies" into line with other cytoplasmic components, especially the plastids of plants, which are, at least in some cases, self-perpetuating, and multiply by direct division without loss of their individuality. Gatenby (1919*b*) in particular has come out squarely with the declaration that:

> . . . . both mitochondria and Golgi bodies are able to assimilate, grow and divide in the cytoplasm somewhat as a protist assimilates, grows and divides in a watery medium. I do not believe that either mitochondria or Golgi bodies are symbiotic organisms, as has been claimed for the yellow cells of Radiolaria, but it seems true that the cytoplasmic inclusions have a marked degree of independence.

This idea of the independence or individuality of chemical substances, expressed in relation to the Golgi apparatus, in a system which is in itself a co-ordinate and indivisable whole, may not make a strong appeal to those of us who are concerned chiefly with the somatic cells of vertebrates in which the networks frequently attain to a high degree of complexity and in which they often undergo hypertrophy without preliminary fragmentation. Under these circumstances, growth by some process of accretion seems more likely to prevail.

8. *Constitution:*

Thus far only objective findings have been discussed which are subject to verification and confirmation. Any logical interpretation is a thousand fold more difficult. It is unsafe to hazard speculations as to what is going on within the cytoplasmic area which exhibits such striking changes. While our methods of detection are so crude, we certainly cannot exclude the probability that the chemical composition of the Golgi apparatus varies to some extent in different cells. Its affinity for silver salts after certain fixatives and the ease with which it may be blackened with osmic acid have already been mentioned. It may be occasionally stained with iron hematoxylin and resorcin-fuchsin. Either it or some of its components are very soluble in alcohol, for unless preparations are dehydrated very rapidly no trace of it remains.

On this unsatisfactory evidence it has been suggested that it is partly lipoid in nature. According to Gatenby (1920*b*): "The constitution of the Golgi apparatus is probably much like that of the mitochondria, i.e., proteid in some way linked with lipin materials." Bowen (1920) is of the opinion that the Golgi bodies in insect spermatogenesis are made up of two components, one staining darkly and the other lightly, and claims that in this respect they resemble mitochondria. This idea of the existence of two components is further extended by Hyman (1923) in his study of the spermatogenesis of *Fasciolaria*. In certain somatic cells, in addition to germ cells, he found the Golgi apparatus to occur in the form of tiny, almost spherical, vesicles in which he was able to distinguish a peripheral "chromophil" substance surrounding a central mass of "chromophobe" material; but there is, however, no good reason to believe that the Golgi apparatus in the somatic tissues of higher vertebrates is heterogeneous in the same sense.

The chief obstacle is that the Golgi apparatus cannot be clearly seen in living, unstained cells when they are examined in approximately isotonic media, that is to say, in mammalian tissues, for Gatenby claims to have observed it in the living cells of invertebrates. Nor has it been possible to stain it specifically with vital dyes, although many have been tried. We are obliged to admit that the Golgi apparatus as revealed in fixed preparations, upon which we have for the moment to rely, is an artifact, in the sense that it conveys an impression which does not fully or accurately represent the actual condition of affairs in the living cell. The dense black outlines suggest a false idea of relative solidity, because it is known that the area occupied by the Golgi apparatus is actually very fluid (see, however, Jordan, 1921). This has been ascertained by carefully crushing the cells so that movements are produced in the cytoplasm, and by the studies of Kite and Chambers in micro-dissection. By means of a special mechanical contrivance, these investigators have been able to make a direct study of the consistency of protoplasm by dissecting cells in hanging drops under oil-immersion objectives (see p. 240). They encountered no areas of resistance suggestive of the presence of a rigid network. Gatenby's (1919*a*) belief that the Golgi rods and granules are denser than the surrounding medium appears to be well substantiated by other investigators working with the same invertebrate material. Herein lies one of the differences which probably exist between the discrete and individual masses of material in lower forms and the complicated networks which are encountered higher up the scale. Since in mammals the fundamental material is not easily displaced by centrifugation, it is safe to assume that it is probably of about the same specific gravity as the remainder of the cytoplasm (Cowdry, 1922).

9. *Relation of the Golgi apparatus to the so-called canalicular apparatus:*

Duesberg (1914, 1920) has reacted strongly against an unqualified acceptance of the conclusion already alluded to (p. 333) that the Golgi apparatus and the clear canals are one and the same structure revealed by different methods of technique. He is of the opinion that the two formations are identical in neurones and non-nervous cells which possess a localized trophospongium (canalicular system), but that in non-nervous cells, with a diffuse trophospongium spread throughout the cytoplasmic area, they cannot be the same, because the Golgi apparatus, on the contrary, is restricted in distribution, being localized at one pole of the nucleus (except in lutein cells).

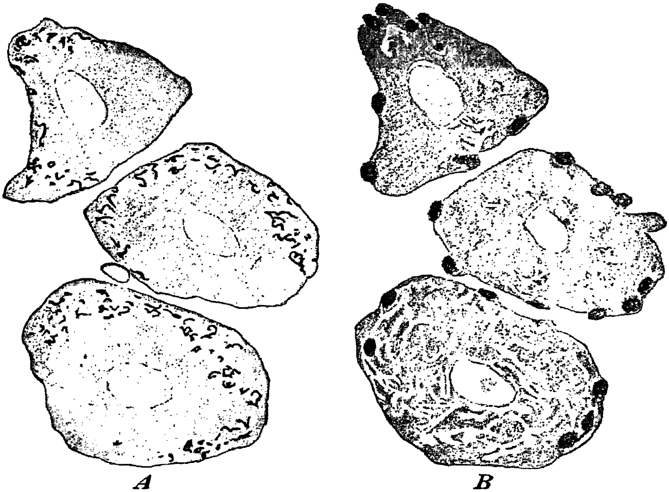

FIG. 29.—(*A*) Spinal ganglion cells of a cat seventeen days after the section of the posterior nerve roots prepared by Cajal's silver method which blackens the Golgi apparatus. (*B*) Same cells after bleaching and staining with iron hematoxylin. The clear canals thus revealed do not correspond to the remnants of the Golgi apparatus (both after Penfield, 1921).

We also note that Penfield (1921) has discovered changes in the Golgi apparatus in injured nerve cells, and that when the same cells were bleached and stained with iron hematoxylin he observed a system of clear canals which in no way corresponded with the remnants of the Golgi apparatus as illustrated in Figure 29. He naturally concluded that the clear canals and the blackened

Golgi apparatus were two entirely different formations. These observations merit very careful consideration. In the first place, it is unusual to have to resort to treatment with iron hematoxylin to reveal canals which when properly fixed are generally visible with almost any or even no coloration of the ground substance. In preparations by similar methods, they are often seen side by side with the blackened masses (Cowdry, 1912). A close examination of his figures shows that the clear canals which he found are not exactly the same as the canalicular apparatus in normal nerve cells. The canals are angular and to some extent suggestive of shrinkage spaces; they are abundant in the peripheral cytoplasm, and in some cases appear as if they might have penetrated into the cell from without; whereas in normal cells of the same kind, the canalicular apparatus presents rounded contours, and is usually situated in the intermediate zone of cytoplasm, leaving a layer of cytoplasm immediately about the nucleus and just beneath the cell membrane clear. This may mean that we are dealing with a canalicular apparatus distinctively changed by section of the nerve roots or that we are confronted by an altogether different type of tubular system.

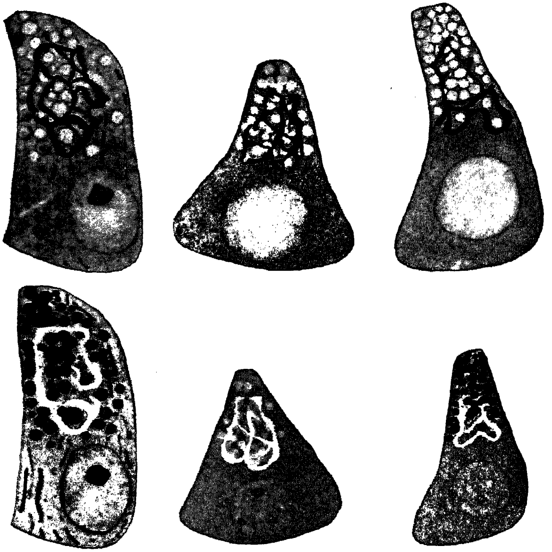

FIG. 30.—Three acinus cells of a guinea pig's pancreas form three specimens, each blackened with osmic acid to reveal the Golgi apparatus. The same cells after bleaching and staining with iron hematoxylin show systems of clear canals exactly corresponding with the blackened networks.

On the other hand, observations are not lacking that there is often a close correspondence between systems of clear canals and blackened networks in normal nerve cells (Cowdry, 1912, 1923). When preparations of the Golgi apparatus in acinus cells of the pancreas are bleached and restained with iron hematoxylin, clear canals are seen in place of the blackened networks (Fig. 30). The hydration and dehydration bring about some shrinkage in the cells, but the canals certainly illustrate faithfully the relative size and shape of the apparatus.

But clear chromophobe spaces in the cytoplasm are not always of the same origin. They may represent areas from which the mitochondria have been dissolved; instead of being restricted to a definite location they may be experimentally produced throughout the cytoplasmic area; in other cells pointed clefts, which are apparently technical artifacts, may be continuous with a canalicular apparatus in its proper location. And these are not the only possibilities that complicate the problem. Repeated attempts on my part to make clear canals artificially in mixtures of gelatin and lecithin, fixed by methods designed to reveal the canalicular apparatus, have not been particularly fruitful. Preparations made in this way and stained with iron hematoxylin contain canals and vacuoles of many sizes. By careful selection, however, it is possible to gather together a series of canals which resemble to some degree the intracellular formations which are so perplexing. That all clear canals are not fixation artifacts may be concluded from Bensley's (1911) observation that they may be seen in living islet cells of the pancreas, but the mere act of taking living cells from the body and of bringing them under the microscope for study may, and probably does, initiate changes in the cytoplasm which may be wholly or in part responsible for their appearance. The same investigator made a parallel study of the clear canals in plant and animal cells by improved methods of technique (1910). Through the study of both living and fixed tissues he found a very significant series of changes in growing cells of the onion tip (Fig. 31). In the youngest cells he discovered a system of clear canals, agreeing in many details with those brought to light by the same methods in animal cells. With increase in age the canals enlarged and finally gave rise to the familiar plant-cell vacuole. On the basis of these observations he suggested "that the network of canals found

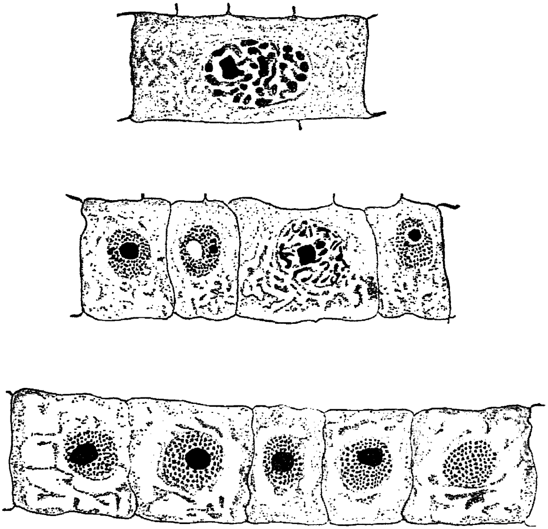

FIG. 31.—Stages in the development of a typical vacuolar apparatus from a system of fine canaliculi in the cells of the root tip of an onion (after Bensley, 1910).

in so many animal cells is the physiologic and morphologic equivalent of the vacuolar system in the plant cell."

The botanists, Guilliermond and Mangenot (1922), have also applied methods for the demonstration of the Golgi apparatus to barley cells (Fig. 32). They seem to have confirmed Bensley's belief that there exists in animal cells an area corresponding to the vacuole in plants. If further work shows that this is in truth the case, interesting and new opportunities for experimental study will be opened up of a kind essentially different from those contingent upon the discovery of the nucleus, and our ideas of the probable constitution of the materials involved will receive some enlightenment.

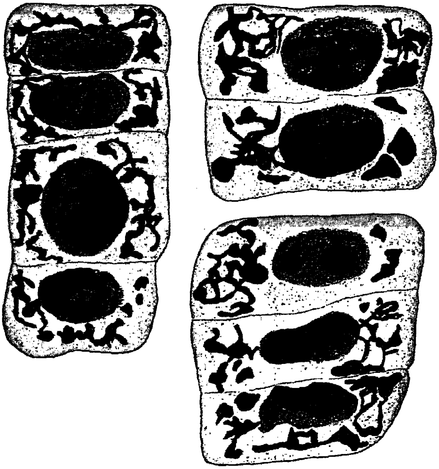

FIG. 32.—The Golgi apparatus in barley cells (after Guilliermond and Mangenot, 1922).

10. *Function:*

In addition to this view that the Golgi apparatus is the homologue of the plant-cell vacuole, several other suggestions have been made. Hirschler's (1918) contention, that it helps to isolate certain cell substances, is supported by Hyman (1923) who remarks that this idea

> . . . . fits in well with observed conditions in Fasciolaria. In the "resting," physiologically active, cell the bodies are diphasic spheres. During mitosis these fragments and both chromophil and chromophobe substances are drawn to the centrioles. The diphasic spheres are then formed again in each daughter cell. It may well be supposed that the function of the chromophil substance is to isolate the chromophobe substance from the remainder of the cytoplasm. The heterogeneity of the cytoplasm will thus be increased and a more orderly interaction of its parts made possible.

This theory recalls some points in Regaud's eclectosome hypothesis of mitochondrial function (p. 324). In higher forms, however, it is the exception rather than the rule to find structural indications of diphasic character. It has also been suggested by Saguchi (1920*b*) that the Golgi apparatus (i.e., "intracellular network") takes part in the act of secretion. The part which it may play in affording a surface for electrical absorption and other phenomena is indicated by R. S. Lillie on page 177.

At present it is unwise to be too specific. Since the whole cell is our unit it is altogether likely that the portion of the cytoplasm which is revealed to us in fixed preparations in the form of the now familiar Golgi apparatus may take part in many different vital manifestations. In the present state of our knowledge it is unsafe to place too much reliance in the idea that it is strictly homologous in different cells, though within limits which cannot yet be defined it may be generally so. The increase in fluidity and decrease in visibility in the living cells of many vertebrates as compared with forms "lower" in the phylogenetic series should also be taken into account. The orderly interaction between relatively simple substances, often of inorganic nature, in the more fluid parts of the cell is by no means to be ignored. Topographically the Golgi apparatus occupies a central position in the cell and its form is probably changing continually as it plays its obscure rôle in the cellular economy. The fact that it is relatively larger in the most active stages of cytomorphosis is significant. Its activities may be bent in one direction during spermatogenesis and along entirely different lines in cells specialized to perform other duties.

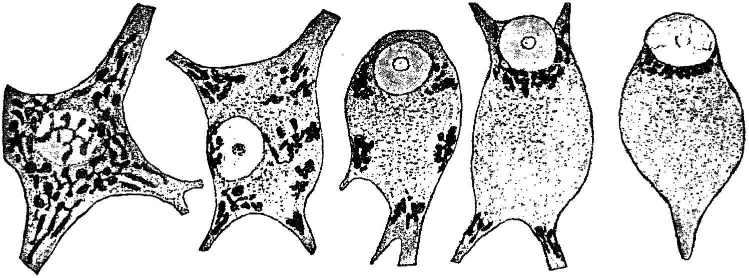

FIG. 33.—Different phases in the disintegration of the Golgi apparatus in motor neurones following operation (after Cajal, 1915).

11. *Changes in pathological conditions:*

It is therefore not surprising that investigators have thus far been unable to bring to light any guiding principle or uniformity in the reactions of the Golgi apparatus to experimentally altered physiological conditions and to injury (see Figs. 33 and 34). For convenient reference the following brief summary is given (p. 347).

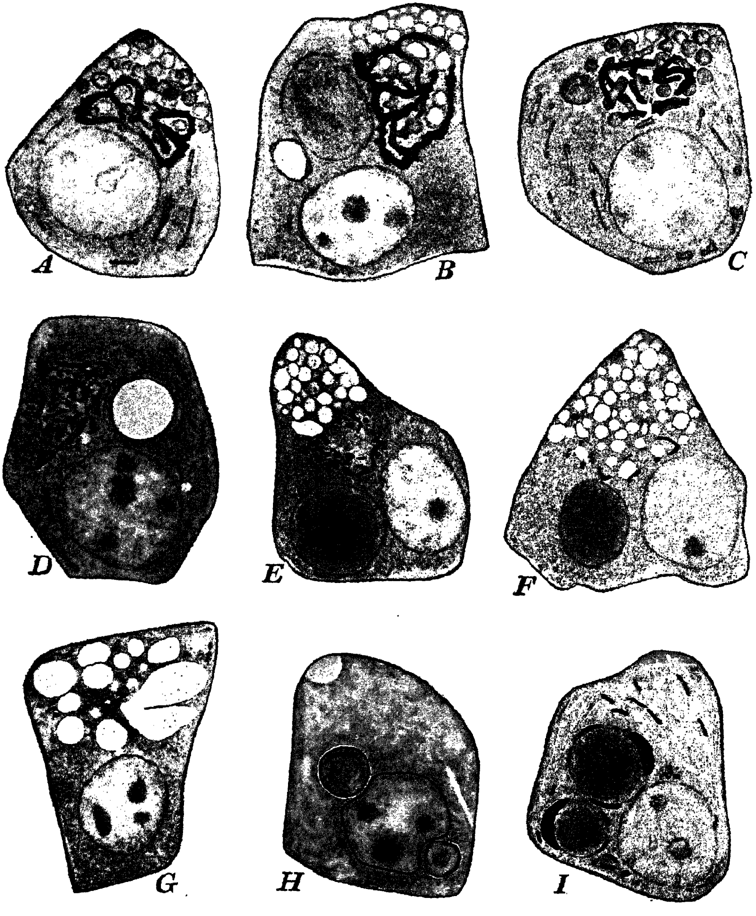

FIG. 34.—The effect of phosphorus poisoning upon the Golgi apparatus in the acinus cells of the guinea pig's pancreas prepared by Kopsch's method of prolonged treatment with 2 per cent osmic acid. *A–H* illustrate the blackening of the Golgi apparatus and its progressive disintegration and final disappearance. *I* illustrates a cell which has been bleached with permanganate and oxalic acid, and counter-stained with fuchsin-and methyl green to show the mitochondria, which are here represented in black.

PATHOLOGY OF THE GOLGI APPARATUS

Monti (1903, p. 187)	Intestinal epithelium in hibernation	Clear canals disappear in hibernation
Holmgren (1904, p. 175)	Variations in metabolic activity of liver cells	Clear canals more prominent after carbohydrate feeding
Martinotti (1904, p. 52)	Muscle cells after tearing out nerve	A preliminary report showing irregularity in morphology
Moriani (1904, p. 630)	Human-breast carcinoma	Large circumnuclear network suggestive of fetal cells
Verson (1908, p. 496)	Tubercular lymph glands and giant cells; hypertrophy of prostate	Fragmentation
Lucioni (1909, p. 459)	Cells of a naevus (human)	Golgi apparatus takes up an eccentric position
Veratti (1909, p. 43)	Adenocarcinoma of intestine;	No specific changes
	Adenocarcinoma of stomach; 3 mammary gland cancers 3 lip cancers 2 bladder cancers 3 sarcomata (all human)	Results uncertain owing to confusion with mitochondria
Sangiori (1909, p. 340)	Experimental toxic nephritis	Illustrations very suggestive of incomplete impregnation and faulty technique (compare Basile, 1914, p. 3)
Taddei (1910, p. 435)	Hypertrophy of prostate	Normal not described so changes uncertain
Marcora (1910, p. 398)	Changes in hypoglossal cells following section and tearing out of nerve	Breaking up into island-like fragments; condensation to one pole of nucleus and granulation. Complete recovery in 55 days
D'Agata (1910, p. 518)	Gastric cells of Triton following operations on stomach	Complete reversal in position
Savagnone (1910, p. 5)	Breast carcinoma Sarcoma of jaw Giant-celled sarcoma	Illustrations unconvincing

PATHOLOGY OF THE GOLGI APPARATUS—*Continued*

Legendre (1910, p. 215)	Electric stimulation of posterior root of lumbar ganglion of dog	Fragmentation with dispersal to periphery of the cytoplasm
	Homotransplantation of ganglia, in rabbit	Pulverization and disappearance
Battistessa (1911, p. 353)	Nerve cells after lead and strychnin poisoning	Reduction in size and condensation. No tendency to dispersal
Cajal (1912, p. 217)	Nerve cells in rabbit in experimental rabies	Fragmentation and pulverization of network
	Cortical cells of cat 3 days after traumatism	Partial confirmation of Marcora
Tello (1913, p. 149)	Epithelioma of tongue	Hypertrophy, fragmentation, and disappearance
	Adenoma of mammary gland	Initial hypertrophy, vacuolar degeneration and loss of staining reaction
	Experimental kieselgur granuloma	Hypertrophy and fragmentation
Fananas (1913, p. 128)	Caseating giant cells	Fragmentation and dispersal to peripheral cytoplasm, leaving clear center, with final loss in staining reaction
Rio-Hortega (1914, p. 114)	Paralytic rabies	Initial hypertrophy with ultimate fragmentation
Basile (1914, p. 3)	Unilateral nephrectomy in guinea pigs	Complete reversal in the position of the reticular material in the cells of the tubuli contorti and recti
Cajal (1915, p. 173)	Effect of pilocarpin on salivary glands and pancreas of cats	Fragmentation
p. 190	Section of medulla	Nerve cells show breaking up of material into islets with migration to peripheral cytoplasm
p. 193	Traumatism of cortex	Atrophy of material with fragmentation but no peripheral migration

PATHOLOGY OF THE GOLGI APPARATUS—*Continued*

Cajal (1915, p. 195	Cells of Schwann following section of sciatic	Same
p. 198	Autolysis	Fragmentation
p. 199	Transplantation of spinal ganglia	Fragmentation and pulverization but no peripheral migration
Pappenheimer (1916, p. 139)	Experimental uranium nitrate nephritis in rats	Complete disintegration and disappearance. Remaining droplets may contain argentophile component
Corti (1920, p. 57)	Intestinal epithelium in hibernation	Slight reduction in size
Penfield (1920, p. 303)	Cat, decerebration, and high section of cord, tetanus toxin and strychnin	Negative
	Section of axone	Dispersal to peripheral cytoplasm and solution
Penfield (1921, p. 77)	Cat, nerve section	Marked difference in response of Golgi apparatus and trophospongium (clear canals)
Da Fano (1921*a*, p. 360)	White rats exposed to cold	Hypertrophy followed by disintegration of Golgi apparatus in nerve cells
(1921*b*)	A variety of transplantable tumors in white mice, rats, and guinea pigs	Golgi apparatus distinctive in almost each type
(1922)	Mammary glands	Hypertrophy, change in position and partial fragmentation during pregnancy
(1923*a*, *b*)	Vitamine B deficiency in mice	No definite changes in nerve cells
Cowdry (1923, p. 4)	Guinea pigs, phosphorous poisoning	Disintegration but no migration of fragments into peripheral cytoplasm

In several instances the observations give the impression of having been rather superficial. On account of the very real difficulty of securing adequate controls, the time has not yet come when human tissues can be profitably investigated. It cannot be too strongly emphasized that vagaries in technique are fruitful sources of error. Sufficient observations have, however, been made to show abundantly that in certain conditions the Golgi apparatus is really very sensitive to pathologic change while, like the mitochondria, it is resistant in others, and to indicate that results of the greatest interest may be obtained through the systematic experimental study of the Golgi apparatus in animal tissues. The general status of this little-known portion of the cytoplasm in cellular pathology is much the same as that of the mitochondria, except that its study is a newer development and suffers from the fact that results cannot be conveniently verified by the observation of living cells.

III. CHROMIDIAL SUBSTANCE[1]

1. *Discovery:*

If we include, as is customary, under this very general heading the Nissl substance of nerve cells and the basophilic material of glands as well as substances observed in Protozoa and in plants, we should have to delve deeply into medical history to find descriptions which might justly be considered to be original. To do so would involve time-consuming and futile controversy as to priority. Our knowledge of these materials has come gradually parallel with improvements in the compound microscope, the introduction of apochromatic lenses, and in technique.

2. *Definition:*

The term "chromidia" has been very loosely used since the announcement of the chromidial hypothesis by Hertwig (1902). Dobell (1909) understands it to mean ". . . . any fragments of chromatin—irrespective of their shape or function—which lie freely in the cell, without being massed together into a definite nucleus." This is perfectly straightforward, but confusion has arisen through the use of slipshod methods in the indentification of "chromatin." Methyl green, which has been repeatedly recommended by morphologists, cannot be fully relied upon because of its lack of specificity; it has, for example, a strong affinity for the granules of mast cells which are of entirely different composition. Moreover, the so-called "volutine" granules stain even more intensely with basic dyes than does true chromatin. These volutine granules have been appropriately called metachromatic corpuscles by Guilliermond (1910, 1918), who gives a good review of the literature. Stress is laid by Minchin (1916) upon the biological behavior of unknown substances in determining their relation to nuclear chromatin. In his phylogenetic studies he compares chromidia to chlamydozoa, which may be not without significance if Lipschütz (1921) and some other epidemiologists are correct in their contention that nuclear material is extruded in vaccinia and similar conditions. Unfortunately, very few investigators have availed themselves of microchemical methods for the identification of iron (p. 49) and of phosphorus. The chromatin, itself, is a substance concerning the chemistry of which we know comparatively little (p. 74). In practice, under the heading of chromidia, we have therefore to deal with a variety of substances which have been hastily grouped together on account of their general affinity for "basic" stains and their supposed relation to nuclear chromatin and for which no special methods of fixation are required. It is a branch of cytology which has developed almost wholly apart from methods for the study of living cells.

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[1] Synonyms: extranuclear chromatin, Nissl bodies (tigroid and chromophile substance), basal filaments of Solger, basophile substance, ergastoplasm, prozymogen (?), etc. The etymology of trophochromidia, idiochromidia, gametochromidia, and several other terms commonly used in protozoölogy has been discussed by Dobell (1909, p. 282).

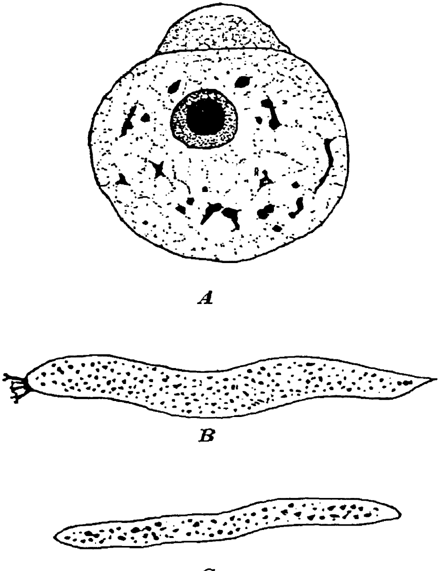

FIG. 35.—(*A*) Chromidia in the cytoplasm of the protozoan, *Stenophora juli*. (*B*) Chromidia forming a diffuse nucleus in the protozoan, *Siedleckia nematoides*. (*C*) Nuclear apparatus in the form of chromidia in *Bacillus flexilis* (after Dobell, 1909).

3. *In the Protista:*

Many papers have been published dealing with chromidia in Protozoa and bacteria (Fig. 35). Chromidia possess no morphologic individuality. In fixed and stained specimens they occur in the cytoplasm of many cells in the form of masses of irregular size and shape; they seem to be derived from the breaking-down of definitive nuclei, as well as from chromatin extruded through the nuclear membrane, and to be endowed with the ability to give rise later to other nuclei (see, however, Kofoid, 1923). When present in non-nucleated cells they are said to constitute a "distributed" or a "diffuse" nucleus (Calkins, 1905). Observations such as these form the basis of Hertwig's karyoplasmic relation hypothesis and of the theory of binuclearity advanced by Schaudinn with the enthusiastic support of Goldschmidt (1904, 1910, etc.), both of which contentions are discussed elsewhere (p. 555).

4. *In the metaphyta:*

The extrusion of nuclear substance into the cytoplasm is described in a long list of papers (see von Derschau, 1914). West and Lechmere (1915) claim that not only does material leave the nucleus but that it is also capable of penetrating through the cell wall and of entry into adjacent cells, but Sharp (1921) is of the opinion that the passage into neighboring cells is probably in many cases a degenerative phenomenon.

5. *In the Metazoa:*

Goldschmidt's (1904, 1910) studies on *Ascaris* are the starting-point of our knowledge of "chromidia" in multicellular animals. In many cells he discovered filamentous bodies (Fig. 36), often coil-like and sometimes definitely grouped about the nuclei, which he believed to be analogous to the chromidia first described by Hertwig in Protozoa. He advanced the view that material of this kind is very widely distributed in animal cells, and attempted to include under this general category the Nissl bodies of nerve cells, the basal filaments of gland cells, and in addition the mitochondria, the Golgi apparatus, and many other cellular constituents which we now know to be of a different nature. This sweeping generalization gave rise to a very active controversy, the early phases of which are best summarized by Duesberg (1912). More recently Kulmatycki (1922), after a careful cytological study of *Ascaris*, has advanced the conclusion that although chromidia are colored by "chromatin" dyes they react rather differently from the nuclear chromatin, and, further, that there is no satisfactory evidence that they arise from the nucleus. He is of the opinion that the chromidia of Goldschmidt rather closely resemble mitochondria, and has christened them by the new name of "Ascaridochondria." Although we must retain an open mind regarding the exact nature of the chromidia in *Ascaris*, we are obliged to admit that it is by no means a rare occurrence for nuclear material to pass into the cytoplasm in large or in small amounts. The transfer, like so many other cellular phenomena, may be seen with almost diagrammatic clearness in insects (Nakahara, 1917, and others). We have, moreover, reliable evidence to show that in higher forms the Nissl bodies and basal filaments of Solger (but not of Altmann) are, as Goldschmidt suggested, to be grouped among materials formed at least in part as a direct result of nuclear activity. It is hardly necessary to say that interaction between the nucleus and the cytoplasm is probably one of the most fundamental of all vital processes (see p. 548 and discussion by Tennent, 1922).

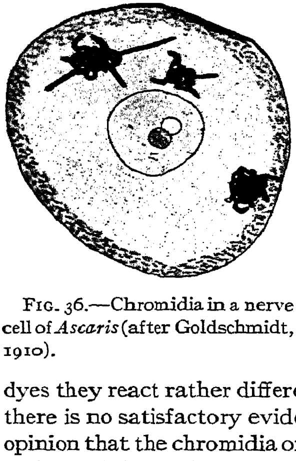

Fig. 36.—Chromidia in a nerve cell of *Ascaris* (after Goldschmidt, 1910).

Much work has been done in the hope that a study of the Nissl bodies in nerve cells would afford clues regarding the problem of the nature of mental activity. The early literature is well reviewed by Barker (1899). Though we may seem almost as far as ever from an understanding of the fundamental processes involved, results of real importance have been obtained. It has been shown, for instance, that not only are the larger groups of nerve cells characterized by the possession of distinctive Nissl bodies when prepared by well-regulated methods, but, further, that the Nissl bodies differ even in cells supplying heart muscle, smooth muscle, and striated muscle (Malone, 1913, 1923). Nissl bodies are still the most useful indicators of pathologic change in nerve cells where they are apparently even more sensitive to injury than the mitochondria (p. 327). They undergo a definite series of alterations following injury and functional exhaustion characterized by loss of distinctive shape (chromatolysis) and ultimate disappearance. It has recently been shown by Nicholson (1923) that parallel changes take place in the amount and distribution of "masked" iron (see Fig. 37).

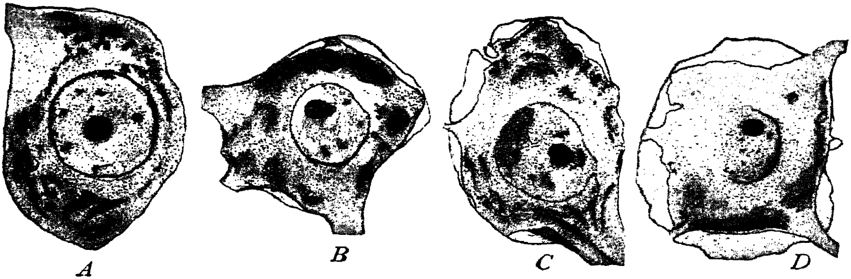

FIG. 37.—Stages in the disappearance of iron-containing protein in the cells of the hypoglossal nerve following axone injury: (*A*) the normal; (*B*) the third day; (*C*) the seventh day; and (*D*) the fifteenth day (after Nicholson, 1923).

Since formed Nissl bodies cannot be clearly seen in living nerve cells, there has always been some doubt regarding the way in which we may rightly interpret these findings. Mott (1915) is probably justified in concluding from his ultramicroscopic studies that the fundamental substance in living cells occurs in a form wholly different from the distinctive Nissl bodies with which we are so familiar, but it does not follow that it actually exists in the particulate state in which he observes it. That is to say, we cannot exclude the possibility that the very considerable mechanical manipulation, which is necessary to bring the cells under the ultramicroscope, in itself initiates some change in the fundamental substance.

It is now common knowledge that to some extent at least the shape of the Nissl bodies depends upon the fixative used. Mixtures of potassium bichromate and osmic acid do not give the sharp and clear pictures which may be obtained by using alcohol, mercuric chloride, acetic acid, and other ingredients. It is a relatively simple matter to choose at will fixatives which will give us large or small Nissl bodies. But when the application of standard methods reveals the existence of different Nissl bodies in neighboring cells of the same preparation, it is quite obvious that a difference of some kind obtains in corresponding living cells. This view, that the substance of the Nissl bodies is present in solution, fortunately in no way detracts from the value of observations which have been and are being made. It only means that we fall back upon hypothetical, but probable, variations in the nature and amount of the original material to explain differences in the shape of Nissl bodies resulting from fixation. For example, cells in which we see large Nissl bodies may differ from others showing smaller ones in the coagulability of the fundamental material. Variations in coagulability may in turn depend upon variations in the character of the material or in its concentration. Thus the loss of shape during the reaction called "chromatolysis" is probably associated with a depletion of the fundamental substance.

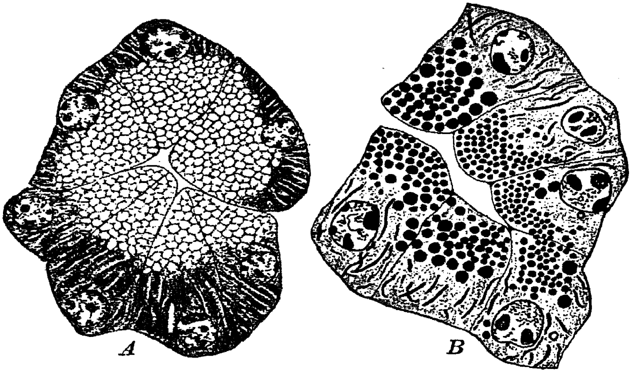

FIG. 38.—The difference in the appearance of acinus cells of the guinea pig's pancreas following fixation in chrome sublimate and staining with toluidin blue (*A*) and after fixation in acetic osmic bichromate mixture and staining with fuchsin and methyl green (*B*). In *A* the homogeneous basophile material is interrupted by clear striations representing the mitochondria which have been dissolved out by the fixative. In *B* the mitochondrial filaments are preserved and are represented in black (after Bensley, 1911) modified.

The basal filaments (i.e., "basophile materials") of gland cells are also artifacts in the sense that the material does not occur in filamentous form in living cells. As in the case of the Nissl bodies, they are best seen after energetic fixatives which have for their chief ingredients: alcohol, acetic acid, mercuric chloride, chloroform, and other strong coagulants. But when fluids like that recommended by Bensley containing only potassium bichromate and osmic acid with a little acetic acid are employed, the basal zone of the cell is seen to be occupied by a diffuse cloud of basophilic substance. Compare *A* and *B* in Figure 38. Chromidial substances which are similar, at least in their more pronounced properties, occur in the cells of many different glands. They have been most carefully studied in the acinus cells of the pancreas for which reference may conveniently be made to extensive contributions by Laguesse (1906) and Bensley (1911). Like the Nissl substance it is relatively insoluble in alcohol, responds to microchemical tests for iron, and cannot be seen in the living cell. As revealed in fixed preparations, it is an equally good criterion of cell activity and cell injury. But here as in many cytological problems our functional interpretations must necessarily lag far behind on account of the great difficulty of projecting accurate methods of chemical analysis into such very small units as the cells.

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[1] For ready reference the literature is given in three parts, relating to the mitochondria the Golgi apparatus, and the chromidial substance. Papers which would fall in the first division are so abundant that, in order to save space, references have been omitted to those published before 1912, since they may conveniently be found in Duesberg's (1912) monograph which is available in most libraries. An effort has been made to include papers published subsequently up to July, 1923. Where, in the text, it has been necessary to make a choice from a large number of references, the most recent contributions have usually been cited, even at the sacrifice of priority, because they are not only more up-to-date but in addition contain useful references to the earlier literature.

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SECTION VII

BEHAVIOR OF CELLS IN TISSUE CULTURES

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BEHAVIOR OF CELLS IN TISSUE CULTURES

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Cell culture, *tissue culture*, and the *cultivation of cells and tissues in vitro* are terms applied to the cultivation, outside the organism, of various types of cells that have been explanted from the Metazoa. The methods are, for the most part, adaptations of some of those long used in bacteriology, such as the hanging-drop and Petri-dish techniques. The hanging-drop method, with which we are more especially concerned, is the one usually in mind when referring to this subject. Tissue culture has already revealed new facts concerning the behavior, structure, and physiology of the living body cells which the older cytological techniques, involving fixation, sectioning, and staining, could not show. It is especially suitable for the study of the living somatic cells.

The first factor which makes tissue culture possible is the ability of cells to survive for varying lengths of time after the animal is killed or after they have been removed from the organism. The length of survival depends upon the type of cell and upon the environment. When small pieces of the various tissues of the adult rat are cut up in sterile Locke solution and kept at 37° F., the large macrophages or clasmatocytes, tracheal cartilage, kidney epithelial, and smooth-muscle cells were still alive at the end of ten days. The epithelial cells of the salivary glands, bladder, trachea, and tongue have survived as long as 192 hours; endothelium, small and large lymphocytes, and microcytes 168 hours, lung epithelial cells 144 hours; leukocytes and Kupffer cells 120 hours; brain macrophages, pancreatic epithelial, liver, and Sertoli cells 96 hours; red blood cells, connective tissue, ovarian follicular, uterine epithelial, and uterine gland cells 72 hours; epididymis epithelial cells 48 hours; and adrenal cells 24 hours. Nerve, skeletal-muscle, and heart-muscle cells survive less than 24 hours. When the entire animal is kept at 37° F. the period of survival is much reduced, and the reduction is still greater when the animals are kept at room temperature. All cells that survive under such conditions develop in their cytoplasm granules, and sometimes vacuoles, which have a marked affinity for neutral red, while the cells that die stain diffusely (Lewis and McCoy, 1922*a*, *b*). Lambert (1913*a*, *c*) found that small pieces of embryonic chick and rat tissues, placed in hanging drops at 7° C. for six days, subsequently showed cell migration when incubated, unless the tissue had been frozen. At 0° C. or 1° C. for 8 days, active migration occurred after a long latent period. If they were kept for more than ten days at this temperature no migration occurred. At 6° to 7° C. for twelve to fifteen days, there was active migration, but after eighteen days there was very little. He claims that at 37° C. cells may live for many days or even for months. Jolly (1910*b*) found that the white blood cells of *Rana temporia* showed amoeboid movements after being kept for nine months in a sealed tube in an ice box. With the ordinary technique used for tissue cultures, where small pieces are cut up in sterile Locke or Ringer solution, there is no difficulty in keeping the cells alive at room temperature until cultures are made.

I. TECHNIQUE

The ordinary hanging-drop technique is comparatively simple. Small pieces of tissue, taken from the living or recently killed animal, are cut up into fine particles, a millimeter or less in diameter, in a small dish containing Locke or Ringer solution. These particles (or explants) are transferred with a pipette to a cover glass, and a small drop of the medium is added. The cover glass is then sealed over a hollow ground slide with stiff vaseline or paraffin and the preparation kept at a suitable temperature. Aseptic precautions must be observed. If the culture is successful the cells migrate from the explant, along solid or semisolid supports, in the medium. The mass of migrant cells, which may or may not increase in number by division, are termed *growth* or *outgrowth*. Tissue culture depends primarily upon two factors, (1) the ability of the various types of cells to survive after the death of the animal or removal from the body, and (2) the ability of the cells to migrate away from the explant into positions where their behavior and structure can be observed under favorable conditions. A third factor, the multiplication of cells, plays an important rôle in many investigations.

II. MIGRATION OF CELLS

Much can be learned from cells that merely migrate, and this phase should be considered apart from multiplication, since it is not dependent on cell division either within the explant or in the outgrowth. Apparently none of the cells so far examined possess organs for swimming, consequently they must have solid or semisolid supports of some kind. In clotted lymph, plasma, agar, and gelatin certain elements in the medium furnish this, while in fluid media the under surface of the cover slip or the surface film of the hanging drop supplies the necessary support. Various other solids introduced into the medium, such as fine silk and cotton threads, spider webs, and glass wool, can be utilized by the migrating cells.

All of the various types of cells that migrate on the under surface of the cover glass in fluid media possess definite adhesive qualities (W. H. Lewis, 1922*d*), and, since practically all types of cells that migrate in plasma or its various modifications likewise migrate in fluid media, it seems safe to conclude that all migrating cells are sticky for the substances on which they migrate. They are sticky because of the very material of which they are composed, just as egg albumen, glue, and mucilage are sticky. If the cell surface is naturally sticky under the conditions of migration, we can scarcely consider that the adhesion of cells to solids is a form of tropism. The term "stereotropism" has frequently been employed to indicate a reaction exhibited by all cells that migrate out from culture explants on solid supports (Harrison, 1911*b*, 1914; L. Loeb, 1912). The fact that cells migrate only on solids does not necessarily mean that they are stimulated by solids as such, nor is there any reason to believe in a sensitiveness of cells corresponding in any way to the response of animals to tactile stimuli. The adhesion of cells to solids is a passive phenomenon but a necessary factor for the process of migration.

Concerning the factors which cause cells to migrate from the explant along the cover glass or other solids, on the surface films of the liquid drop, or on other cells (which behave as solids) there is still much uncertainty. The work of Tait (1918) and Leo Loeb (1920) indicates that the progressive movements of the cells may depend upon local variations in the metabolism which produce local and alternating variations in surface tension. Burrows (1913*a*) suggests that the acid formation within the explant, in contrast to the more alkaline medium on the outside, may, by altering the surface tension on the two sides of the cell, force it out from the explant. The sheets and networks of migrating cells seem to be under considerable tension, as slight disturbances are often followed by marked retraction toward the explant. It is possible that the surface film between the fluid medium and the solid may exert a definite pull on the cells, causing them to migrate outward under tension.

All migrating cells exhibit changing pseudopodia varying in size and shape; even liver, intestinal epithelial, and striated-muscle cells, which under normal conditions are supposed to be without them, may show quite marked pseudopodial formation.

Under the term pseudopodia we include all the various types of cytoplasmic projections or processes that come and go, sometimes rapidly, sometimes slowly, according to conditions and cell type. They vary from broad, thin sheets to long, extremely slender threads and amoeboid types. Cell migration is dependent upon, but does not always follow, their production. Often no sharp line can be drawn between the behavior of the advancing broad, thin edge of flattened cells and of the long slender tips of spindle-like cells and the behavior of the pseudopodia. Such cells appear to glide along. Mesenchyme, endothelium, muscle, and epithelium often migrate in this fashion. These advancing edges and points are as truly pseudopodia as are the amoeboid-like ones of the white blood cells. Leo Loeb (1921*a*) finds that the migrating amoebocytes from *Limulus* blood have threadlike pseudopodia in a hypertonic medium, sharp, tonguelike, and broad ones in isotonic, broad ones in hypotonic, and balloon-like ones in a very hypotonic medium. Such variations are probably dependent upon alterations in the consistency of the cytoplasm; consequently, the form of the pseudopodia of the various types of cells in an isotonic medium may indicate differences in their consistency. The ordinary multipolar embryonic mesenchyme cells often possess very long, threadlike pseudopodia, while the ordinary lymphocytes and endothelioid cells of adult human lymph nodes usually have broad pseudopodia. The cytoplasm of the mesenchymal cells, or at least that part which goes into the formation of the pseudopodia, is probably firmer in consistency than that of the lymphocytes. Other factors than the density of the medium play a part, e.g., its H-ion concentration, the presence of various substances, and the metabolic condition of the cell. Variations in the form of the pseudopodia as great as those produced by variations in the density (tonicity) of the medium occur at different times in the same cell without change of medium. The most notable example of this is to be seen during mitosis (Levi, 1916*d*). The ordinary embryonic mesenchyme cells, as already noted, possess, during the resting stage, long, slender pseudopodia. During the prophase and metaphase most of these retract into the body of the cell or become very thin. During telephase numerous very active, short, broad, bleb-like pseudopodia appear and disappear with considerable rapidity. In mesenchyme cells one encounters all gradations from broad, flat plates to slender threads. The clasmatocytes often possess thin, wavy, or curled membranous pseudopodia which attach themselves here and there to the cover glass or may be entirely free. Pseudopodia vary greatly in their activity according to the cell type, the condition of the cells, and the medium. Some change from second to second or minute to minute, others change very slowly during many minutes or even hours or days.

1. *Saline fluids* (*artificial media*):

Embryonic chick cells migrate in a great variety of fluid media. Mesenchyme and endothelium, for example, migrate and survive for several days in a simple 0.75 to 0.9 per cent solution of sodium chloride. In a solution with 0.9 per cent NaCl and 0.00625 to 0.125 per cent $CaCl_2$ the extent and amount of migration, as well as the duration of the life of the cells, is increased. In a solution with 0.675 per cent NaCl and 0.105 per cent KCl the numerous migratory cells may survive for five days. In combinations of NaCl, $CaCl_2$, KCl, and $NaHCO_3$ the percentages of the various salts may vary considerably, yet migration may be fairly extensive and the cells survive for from two to nine days. The percentage of NaCl may be decreased to 0.45 per cent or increased to 1.6 per cent and considerable migration result, provided the other salts are retained in the usual proportions given for Locke solution (Lewis and Lewis, 1911*b*). Migration is more extensive, mitoses more abundant, and duration of life longer in a Locke-bouillon-dextrose medium (Locke solution [NaCl .9 per cent, $CaCl_2$ .025 per cent, KCl .042 per cent, $NaHCO_3$ .02 per cent] 80 per cent, chicken bouillon 20 per cent, dextrose .25–1 per cent). If this be diluted with distilled water (3:2), so that the sodium chloride content is 0.54 per cent, migration begins earlier and is more rapid than in controls, but the duration of life is shorter. If the Locke-bouillon-dextrose medium is made hypertonic by evaporation until the NaCl has a concentration of 1.5 per cent, migration may not begin until the second or third day instead of within a few hours, as in the control (Hogue, 1919).

Drew (1922, 1923*a*) has recently devised a modified Locke solution ($NaCl$ .9, $CaCl_2$ .020, KCl .042, $NaHCO_3$ .020, $MgHPO_4$ .010, $CaH_4[PO_4]_2$ .010), in which the calcium salts are probably in the colloidal state. To this he added embryonic extract, autolized-tissue extract, or tumor extract, and obtained excellent cultures of embryonic, adult, and tumor tissues that were propagated for a considerable time by subcultures. Various other artificial media have been devised, such as Locke or Ringer solution with gelatin, agar-agar, egg albumen, or peptone (M. R. Lewis, 1911; Lewis and Lewis, 1911*a;* Smyth, 1914*a;* Swezy, 1915). Except in Drew's saline extract medium, adult cells migrate only feebly or not at all in these various saline fluid media. The fluid media have certain advantages over clotted plasma and lymph media for cytological studies, in that the cells migrate out along the cover glass and flatten out so as to become very favorable objects for study with the highest powers. The fluid drops are also much easier to manipulate; vital dyes and other reagents can be added more readily and the reaction of the cells followed under the high power more easily than in plasma.

Attempts to make use of the amino acids in fluid media were made by Burrows and Neymann (1917), but they tended to inhibit cell growth and were apparently not utilized by the cells. Our experience with amino acids has been somewhat similar. Dextrose has an important influence upon maintaining the cells in a normal condition, and it seems probable that they are capable of utilizing this substance (M. R. Lewis, 1922).

2. *Lymph and plasma:*

Harrison (1907, 1911*a*), who first used this method for growing tissues, employed frog's lymph for embryonic frog tissue. Burrows (1910*a*, 1911), working with Harrison, developed the blood plasma medium, using chicken plasma for embryonic chick tissues. It was soon discovered that adult tissues, as well as embryonic, could be cultivated in plasma from a different species (heteroplasma) almost as well as in the plasma from the same animal (autoplasma) or from another animal of the same species (homoplasma). Lambert and Hanes (1911*g*) found that rat spleen could be cultivated in plasma from the mouse, guinea pig, rabbit, pigeon, dog, or man, but not in goat plasma. Embryonic chick tissue can migrate in human, rabbit, or dog plasma, but most tissues seem to grow better in auto- or homoplasma than in heteroplasma. In diluted plasma (three parts to two parts distilled water) growth is accelerated and more extensive than in normal plasma (Carrel and Burrows, 1911*e*). Lambert (1914*b*) found that the relatively small, actively motile cells from the bone marrow and spleen showed increased migration in diluted plasma, while cells of the intestinal epithelium, rat-embryo skin, and mouse carcinoma were not influenced by the dilution of the medium; the migration of connective-tissue cells was only slightly increased. Dilution of plasma with isotonic NaCl solution, Ringer solution, Locke solution, or serum produces increased migration, according to Burrows (1913*b*). The amount of growth varies inversely with the age of the animal from which the plasma is taken (Carrel, 1913*b;* Carrel and Ebeling, 1921*b*). Migration and cell multiplication, especially the latter, are increased by the addition of embryonic juice to the plasma medium (Ebeling, 1913; Carrel, 1913*b*). Homogenic and heterogenic blood serum has been found to be a useful culture medium for embryonic chick tissues (Carrel and Burrows, 1911*g;* Ingebrigtsen, 1912*a*). Maximow (1922, 1923*a*) found that the addition of bone-marrow extract to plasma produced marked differences in the behavior of certain types of cells and greatly increased the longevity of the fibroblasts in subcultures.

3. *Influence of hydrogen-ion concentration:*

In the Locke-bouillon-dextrose medium the H-ion concentration may vary between 6.4 and 7.2, but the one most frequently used in our studies is about 6.8, as shown by Lewis and Felton (1921). Tissues exhibit growth when explanted into a solution having an H-ion concentration between 5.5 and 9, but the medium most favorable for growth is one of about pH 6.8 or 7. Tissues explanted into media pH 4–5.2 do not exhibit growth, and those in more alkaline solutions than pH 9 seldom grow. By means of Felton's (1921) drop method it is possible to measure the alterations in the H-ion concentration of the medium produced by the cells growing in it. Cultures that fail to grow in a normal medium pH 6.8 are usually found to have become acid, while those exhibiting extensive growth are nearly neutral. Regardless of the H-ion concentration of the medium in which the tissue has been explanted, it has been found that cultures exhibiting vigorous growth tend to change the pH of the medium toward the neutral point (pH 7). The final H-ion concentration of the cultures in our solution depends upon the amount of dextrose present in it; cultures in solutions to which no dextrose has been added are about pH 7.2–7.6, while those in solutions containing 2–5 per cent dextrose are about pH 6.4–5.6.

In plasma media, according to Fischer (1921*a*), the rate of growth is markedly modified by slight alterations in the hydrogen-ion concentration. The optimum he finds is from pH 7.4–pH 7.8, although the tissue may grow for four to six generations in plasma pH 5.5, or for as many as ten generations in plasma pH 8.5.

Mlle Mendéléef (1923) claims that for the growth of embryonic guinea-pig tissue the most favorable H-ion concentration of plasma is brought about, not by the addition of acid, but by making the plasma acid by freezing it, or by injecting milk into the animal from which the plasma is taken. She finds that the embryonic tissue has an H-ion concentration of 5.8–6, and plasma to be most favorable should have about the same.

Rous (1913) colored plasma blue with litmus, and then explanted into it small fragments of chicken tumor and late embryonic tissue (twenty days). All the pieces were at first colored blue, but as growth began the proliferating pieces soon became pink, showing that they were acid, while the pieces that did not grow remained blue. Levaditi and Gabrek (1914) claim that when pieces of tissue are stained with neutral red and explanted in plasma, those that become yellow (alkaline) are sterile, while those that grow become colorless after one or two generations.

III. TYPES OF CELLS CULTIVATED

Cells from various species of animals have been cultivated, such as man, rat, mouse, guinea pig, rabbit, cat, chicken, fish, amphibia, arthropods, insects, and the sea anemone.

Embryonic cells migrate out much more readily than do adult cells, and practically all types of embryonic cells, especially those of the chick, have been cultivated. The younger the embryo the more readily will the cells migrate. In the Locke-bouillon-dextrose medium, liver cells, for example, migrate more readily from explants of seven-day embryos than from explants of fourteen-day embryos, and there is little or no migration of these cells from explants of sixteen- to twenty-one-day embryos. The older the individual the fewer types of cells that migrate, either in fluid or in plasma media. It would hardly be correct to state that the more highly specialized cells lose their ability to migrate more rapidly than the less specialized ones, since the adult connective-tissue cells or lymphocytes, which have been cultivated, are in one sense as highly specialized as adult liver or pancreas cells, which have not.

Among the embryonic cells there is considerable variation in the readiness with which they migrate. Mesenchyme is the most common and most easily cultivated; endothelium, especially from the liver, endoderm from the stomach and intestine, ectoderm from the skin and amnion, smooth muscle from the amnion, and liver cells migrate with considerable facility; likewise the spleen cells and clasmatocytes. The pancreas and the cartilage cells, on the other hand, even from very young embryos, migrate but rarely in a Locke-bouillon-dextrose medium. Heart muscle, at the most favorable age, migrates out in only about 10–12 per cent of the cultures, while skeletal muscle yields a larger percentage of growth. With both types, however, the number of cultures showing migrating muscle decreases rapidly with advancing development.

Comparatively few types of normal adult cells have been cultivated and then only in plasma, lymph, or their modifications, and in the Drew saline medium plus embryonic, tumor-tissue, or autolyzed normal adult tissue extracts (Drew, 1923*a*). Ectodermal, connective-tissue, spleen, lymph-node, bone-marrow, endothelial, and thyroid cells about complete the list. The most plausible explanation of the decrease in the migratory power of older embryonic and adult cells is to be found in the increasing consistency of the cytoplasm.

IV. GENERAL CHARACTER OF OUTGROWTHS

In the ordinary plasma and fluid media the general character of the outgrowth varies with and is more or less characteristic for each cell type. It is now possible in most instances to identify the outgrowth, that is, the specific type involved, by the patterns formed by the cells in their relation to one another. Mesenchyme, endothelium, and smooth muscle form reticular outgrowths that are sometimes, but not always, distinguishable from one another. Heart muscle migrates as a coarse reticulum and occasionally as a membrane. Skeletal muscle is usually characterized by long, multinucleated strands that often branch and adhere to neighboring ones. Epithelial cells, such as ectoderm, endoderm, liver, thyroid, and kidney produce more or less characteristic membranes or sheets. The characteristic cells of the blood, lymph nodes, spleen, bone marrow and thymus, and also the clasmatocytes, migrate as discrete, isolated individuals which unite only under very abnormal conditions.

V. INTERACTIONS BETWEEN DIFFERENT TYPES OF CELLS

Various combinations of embryonic cells, such as mesenchyme, endothelium, smooth muscle, heart muscle, skeletal muscle, endoderm, ectoderm, liver, kidney, spleen, etc., have been cultivated together in the same hanging drop without any noticeable influence of one type on another, except where the mesenchyme is of sufficient thickness to form a framework, in which certain types of epithelial cells may differentiate into tubules. Without it the cells spread out in sheets. This framework of mesenchyme acts in a mechanical way, rather than by any effect of its secretions. Liver cells behave exactly the same in pure cultures as they do in cultures containing endothelium, spleen, or intestine. Cells from such diverse species as fish and fowl can be cultivated in the same drop (Dederer, 1921), each type retaining its own peculiarities and migrating independently of the other type. These same conditions apparently hold in subcultures as well as in primary ones. Ebeling and Fisher (1922) cultivated together a pure strain of epithelium and one of fibroblasts for two months by repeated subcultures, and found at the end of that period that each type had retained its own characteristics. The epithelial cells still adhered together in sheets, except where the fibroblasts were thick enough to permit tubule formation. The fibroblasts formed the usual loose reticulum.

VI. CHARACTERISTICS OF MIGRATING EMBRYONIC CELLS

1. *Mesenchymal and mesothelial cells:*

The ordinary mesenchyme or connective-tissue cells are the most common and often the most abundant type of cell encountered in the usual run of cultures of embryonic tissues. These cells vary much in shape, from simple spindle or triangular to many-branched multipolar or broad, flat, platelike forms (Plate I *a* and *b*). The various factors determining these differences have not been worked out. The age of the embryo, the medium, and the character of the support on which the cells migrate probably all play their part.

Mesenchyme cells begin to migrate fairly early after the culture is made, and are often the most persistent cell type, especially in subcultures, and give the characteristic radial appearance of migrating cells. They are commonly described as forming a syncytium similar to that supposed to exist in the organism. There are many reasons, however, for believing that they form an adherent reticulum rather than a syncytium (Lewis, 1922*a*). The first mesenchyme cells which migrate out often become entirely isolated from one another, but as the numbers increase they become more or less crowded together and in many places it is impossible to determine how they are attached to one another. In the thinner parts of the outgrowths and at the periphery, where the cells tend to become more widely separated or even entirely isolated, one can watch their slow shifting from one position to another, and can follow the withdrawal of processes, which were adherent or in contact with the processes or bodies of neighboring cells or the cover glass, and the formation of new processes which extend out to the same neighboring cell, to other cells, or to the cover glass. As cells become more and more separated at the periphery of the growth, they may lose all connection with one another, their processes extending out and ending on the cover glass. When the outgrowth is scanty, the majority of the mesenchyme cells may be isolated from the very beginning of migration. It is possible to cause the cells to withdraw all their processes, round up, and lose all connection with neighboring cells in cultures where it would otherwise be impossible to determine between adhesion and fusion. Degenerating cells sometimes behave in a similar manner. Even in places where mesenchyme cells are closely connected, no interchange of granules or mitochondria can be observed. These cells thus behave as individual units that are adherent to one another because their surfaces are sticky.

The transformation of mesenchymal reticulum, consisting of bipolar and multipolar cells, into a typical flat mesothelial membrane, where many of the cells abut one another along lines that show a liberal amount of cement substance with silver nitrate, has been observed in cultures of mesenchyme from chick-embryo hearts (W. H. Lewis, 1923*a*). One can actually watch the partial retraction of long, overlapping processes and the spreading out of the intervening cell edges to meet those of neighboring cells. Since the reverse process also takes place, the changes are in the nature of a transformation of form rather than of a differentiation. The mesothelial cells often show peculiar long striae similar to those seen in endothelium and smooth muscle (Plates I *c* and V *b*). These are not permanent structures, and are probably the result of tension.

2. *Clasmatocytes:*

Cultures from most of the embryonic chick and mammalian tissues show varying numbers of migrating clasmatocytes among the other types of cells.

They appear and remain as isolated individuals, and are characterized by their irregular, changing form and the presence, under normal conditions, of numerous granules and vacuoles that have a marked affinity for neutral red (Plate IX *d*, *e*, and *f*). These cells possess peculiar broad, wavy, membranous protoplasmic pseudopodia, often free from any external attachment, which project into the fluid medium and there wave back and forth while undergoing changes in shape. The attachment of these thin, curled, membranous pseudopodia to the cover glass is usually along a slender, curved line that represents only a small part of the membrane. This line of attachment is more easily seen than the thin dependent part, so that the cells appear to have finger-like processes. The membranous processes are large when the cell bodies are more or less compact and small when the cells are very much spread out into irregular forms. The clasmatocytes often remain more or less fixed in position after a short migration, but even then active changes of their pseudopodia may continue. When spread out into a thin layer a small centrosphere, much like that seen in degenerating cells, becomes visible. These cells possess marked phagocytic powers.

3. *Endothelial cells:*

In ordinary cultures of tissues containing both endothelium and mesenchyme, it is difficult or impossible to distinguish between these two types of cells after they enter the migratory zone. Maximow (1916*a*) found that the capillary vessels in the explant remain unaltered among the other elements of adult connective tissue and give rise to new sprouts, and endothelium that differs more or less markedly from the fibroblasts can be found, even after the first subcultures. After three or four days in vitro, however, this sharp difference may disappear, the vessels become indistinct and separate endothelial cells migrate away, become indistinguishable, and, according to Maximow (1922), become changed into fibroblasts. In one and the same culture some of the endothelial cells may retain their specific character, while in other places they change to a fibroblastic character. It may be that conditions are quite different in embryonic kidney explants, for Rienhoff (1922) has shown that the capillaries and sinuses are differentiated *in situ* from the undifferentiated tissue making up the bulk of the metanephric body of the eight-day chick embryo. There is not only differentiation of the endothelium, but direct growth by sprouting from both the capillaries and the larger sinuses. Rienhoff likewise observed, in the thicker part of the migratory zone, the development *in situ* of sinuses, blood islands, and capillaries when the necessary amount of undifferentiated tissue had migrated out. Usually the outgrowth of the already differentiated endothelium was coarsely reticular in character, and in fixed cultures the cells were often fibrillated and connected by long branching processes. Such outgrowths did not give rise to formed blood-vascular elements. In the thicker parts of the migratory zone capillary sprouts grew out in arciform loops from other capillaries already present in the explant. We have occasionally noted in Locke-bouillon-dextrose cultures, when the migratory zone next to the explant had attained a sufficient thickness of mesenchyme cells, that capillary sprouts, with distinct lumina and sometimes containing blood cells, project from the explant. In the thinner part of the migratory zone the endothelial cells migrate off and become indistinguishable from the mesenchyme. A certain thickness of mesenchyme seems to be necessary, then, for the development of capillaries. The mesenchymal reticulum forms a sort of framework either for the outgrowth of capillary tubes or for the differentiation of more primitive cells into endothelial tubes.

In cultures of embryonic chick liver (W. H. Lewis, 1922*b*), the endothelial cells migrate out along the cover glass (Plates II *a* and IV *a*) or on the surface of the hanging drop in the Locke-bouillon-dextrose medium as an adherent reticulum, somewhat different in character from the ordinary mesenchymal reticulum, yet resembling the latter in so many respects that one would be unable to distinguish between the two types of cells were they mixed together in the same culture. This does not necessarily mean that endothelial cells actually change into fibroblasts. Isolated endothelial cells often migrate off by themselves, and under certain conditions the cells of the reticuli retract their processes and tend to round up, losing all connection with neighboring cells, indicating that the cells are adherent and not fused into a syncytium.

The outer layer of the otherwise homogeneous cytoplasm often shows quite marked longitudinal striae or fibrillae that are more apparent in fixed than in living cells. These striae are not permanent structures and are probably produced by the peculiar manner in which the cytoplasm gels under tension. In young cultures the endothelial cells contain long mitochondrial threads of varying length, sometimes branched and usually having no definite orientation. As the cultures get older the mitochondria tend to become more or less radially arranged about the centriole and enlarging centrosphere and to break up into rods and granules. Granules and vacuoles which have a marked affinity for neutral red gradually accumulate and become concentrated about the region of the centriole and enlarging centrosphere. In the older cultures, where the degenerative changes are advanced, the nuclei often show budding and splitting into several pieces.

4. *Smooth-muscle cells:*

Migrating smooth-muscle cells are readily obtained from the amnion of the chick embryo (Plates III *a*, IV *b*, *c*, and V *a*). They form adherent reticuli that show considerable variation in density, from fairly compact masses to very loose ones, where individual cells may become entirely isolated, as at the periphery of the outgrowth. The cells vary greatly in shape; near the explant they tend to be elongated and bandlike and sometimes exhibit rhythmic contraction, while at the periphery, where the cells are large and flat, there is seldom any *contraction* (M. R. Lewis, 1920*b*). The cells forming the reticuli are attached by overlapping or by processes which behave very much like those of mesenchyme cells. The processes that are adherent to neighboring cells may be withdrawn and new adhesions formed, or the cells may lose all connections with their neighbors. There is more reason to look upon the outgrowth as an adherent reticulum than as a syncytium. There are, of course, many places where one cannot determine this with any degree of certainty. The cytoplasm of the smooth muscle cells is more refractive than that of mesenchyme or endothelium, and on fixation often gives rise to typical myofibrils. The living cytoplasm, with either bright or dark field, appears free of fibrils and homogeneous in the resting cell. Very much elongated overlapping processes may simulate fibrils when parallel to the long axis of the cell. In many places the cells seem to be spread out under considerable tension, and it appears to be along the lines of tension that the contractile substance coagulates into fibrils of various sizes. Sometimes in a cell which is spread out irregularly these fibrils extend in two or three directions (Plate IV *c*).

Champy (1913*a*) finds that smooth-muscle cells dedifferentiate after division into a common indifferent type. We have not observed this in cultures of the smooth muscle of the amnion, and M. R. Lewis (1920*b*) has shown that the forms which Champy finds in fixed preparations are due to the fact that the fibrils do not coagulate upon fixation in the rounded-up metaphase but do appear again in those cells fixed after division is completed. In cultures of the intestine, however, where considerable smooth-muscle migration is to be expected, it has not been possible, as a rule, to distinguish smooth muscle from mesenchyme. Whether this is due to the cultural conditions or to lack of growth of the smooth muscle is not known.

The mitochondria are usually abundant and threadlike. In the older cultures the cells show the usual accumulation of granules and vacuoles which have an affinity for neutral red, the breaking-up of the mitochondrial threads into rods and granules, and the formation of a large centrosphere.

5. *Heart-muscle cells:*

The migration of heart muscle has been obtained so far only from embryonic and very young animals (Plate VI *a*, *b*, and *c*). Burrows (1910, 1911, 1912*a*, *b*) states that the muscular elements of very young embryonic chick hearts migrate out in plasma cultures in the form of "short chains of striated cells which contract rhythmically along with that portion of the heart from which they appear." He found they usually began to migrate between the second and sixth days and that their activity sometimes continued for six to eight days, occasionally as long as twelve days. Very rarely a single isolated heart-muscle cell became separated from the others and beat with a rhythm of its own, different from that of the explant or the rest of the outgrowth. Only a small percentage of his cultures showed any heart muscle.

The identification of heart-muscle in cultures has offered considerable difficulty, and we are uncertain whether Congdon (1915) really obtained heart muscle in the cultures of the ventricles of four- to eighteen-day chick embryos in plasma. Lake (1916) concluded, from heart cultures of young and embryonic rabbits in a medium of three parts plasma and two parts distilled water that most of the so-called "fibroblasts" are muscle cells. His conclusions are probably entirely unwarranted, as are the similar ones of Drew (1922), who cultivated embryonic mouse hearts in a saline solution plus embryonic extract. We have recently cultivated mouse-embryo hearts in the Drew saline embryonic extract medium and embryonic chick heart in plasma, and find, as Burrows did, that the percentage of cultures with migrating heart muscle is very small, most of the migrating cells being either mesenchyme or endothelium.

Levi (1916*e*, 1919*a*), who has given the most complete account of the cytological characters of the cells of the embryonic chick heart in plasma cultures, considers the large, coarse reticular masses as heart muscle and as syncytial in structure. His figures and descriptions correspond more to mesenchyme or endothelium in our cultures of embryonic chick hearts in plasma and in the Locke-bouillon-dextrose medium. Heart muscle, when it does migrate out, is usually so characteristic that one can scarcely confuse it with mesenchyme.

We have made over 3,000 cultures from the hearts of chick embryos varying in age from three to eleven days. About 8 per cent of the cultures from six- and seven-day embryos, 11 per cent of those from four-day embryos, and 2 per cent of those from eleven-day embryos showed migrating muscle. Heart muscle does not usually begin to migrate until the third or fourth day, but may occasionally appear as early as twenty-four hours. Migration is slow and in the form of a coarse reticulum or sheet. The growth never extends out as far as the mesenchyme but usually survives longer than the latter. Although in most places it is impossible to decide between adhesion and fusion, owing to the complexity of the outgrowth, a number of facts leads us to believe that the migrating heart muscle is not a syncytium, but consists merely of adherent cells forming a reticulum or occasionally a membrane. Isolated heart-muscle cells occur. Silver nitrate preparations of suitable cultures show complete cell borders, especially in the membranous type of outgrowth. Some of these membranes had previously been pulsating, so there can be no doubt as to their identity. Individual cells within the muscle reticulum may pulsate at rates different from those of their neighbors, or a single cell may pulsate while the others remain quiescent.

Concerning the structure of the heart-muscle cells, also, we have come to conclusions not in accord with the usually accepted view, namely, that the myofibrils of fixed material do not represent structures present in the living cell. We have been unable to satisfy ourselves that the living muscle cells in our cultures contain fibrillae, although the fixed cells often show them. In this we are not in agreement with Levi (1916*e*, 1922*b*). There are three conditions resembling fibrillae, which are often observed, not only in heart-muscle cells, but in smooth muscle, endothelium, and mesothelium as well; namely, a linear arrangement of long mitochondria, an overlapping of long, slender processes, and tension striations. The latter deserve most careful consideration, because they resemble closely the appearance of fibrillae in fixed cells. Migrating cells become more or less flattened out on the solid supports under considerable tension. The direction of this tension appears to be in line with the cell processes, as though the latter produced a pull on the ectoplasmic layer. The visible striae of various widths and lengths thus produced in the living cell are not permanent but may disappear and new ones may appear in line with new processes. The exact significance of these striations is uncertain. They may extend across the nucleus, indenting it or almost cutting it in two. They seem to be a phenomenon produced by tension and reversible when the tension is altered or relaxed. On fixation they may retain their identity and stain more deeply than the rest of the cytoplasm, resembling myofibrillae. The latter occur, however, in fixed cells which do not show tension striae. The coagulation of optically homogeneous contractile protoplasm into fibrillae of various sizes, down to the limits of visibility, suggests to us that contractile substance possesses a polarity due to the orientation of its colloidal molecules or aggregates, and that only such theories of contractility as are based on changes in the molecular condition can approach validity. Rhythmic contractions frequently occur in heart-muscle cells that on fixation show no fibrillae. Such cells are often irregularly rounded, and the shortening in one direction and swelling at right angles to this is not so obvious as in the normal elongated cell, probably because the polarity of the contractile molecules has become more or less disarranged during the migration and spreading-out of the cells on the cover glass. If we were to venture a crude hypothesis to explain contraction, it would have as its basis the alteration in form of an elongated molecule or aggregate into a shortened, broadened one, due to electrical, physical, or chemical change.

Occasionally, heart-muscle cells are beautifully cross-striated (Plate VI *b*), but most of them, even those which pulsate rhythmically, are not, although at the time the cultures were made those in the explant were cross-striated. Are we to explain this as a dedifferentiation, or do the cross-striations represent one of the states of a reversible contractile substance?

One of the most characteristic features of migrating heart muscle is the presence of glycogen, which is readily revealed during the early stages of iodine fixation. This is present, not as small, round granules, but as large areas of a port-wine colored substance in the cytoplasm.

6. *Skeletal-muscle cells:*

The plasma cultures of Sundwall (1912), Congdon (1915), and Levi (1916*e*) apparently did not show very extensive migration of skeletal muscle elements, but abundant migration often takes place in simple Locke solution (Plate VII *a*, *b*, and *c*) with or without the addition of other substances (Lewis and Lewis, 1917*b*). Explants from seven- to twelve-day chick embryos, containing striated muscle fibers, usually show projecting muscle buds by the end of the first day or during the second. Their maximum growth is not attained until the end of the third or fourth day, and they often remain in fairly good condition until after the surrounding mesenchyme has degenerated and died. In some places one can see that the muscle buds are continuous with the cut ends of striated fibers within the explants. The migrating muscle, however, rarely shows cross-striations, and there is an abrupt transition from the cross-striated part in the explant to the non-striated outgrowth. Occasionally, preparations contain several fibers which are cross-striated throughout their length, except at the protoplasmic ends. The cross-striations may be confined to thin, longitudinal bands or extend partially or completely across the fiber. The muscle buds gradually become more and more elongated, and may or may not retain connection with the fibers in the explant. Sometimes neighboring fibers form plexuses. Fibers with one to several nuclei may migrate away and lose all connection with other fibers, sometimes appearing as large, multinucleated cells. The cytoplasm is more refractive and appears to be denser than that of the mesenchyme, and often shows what appears to be a very fine longitudinal striation, quite different, however, from the myofibrils of fixed normal tissue. This striation is partly due to many longitudinally arranged, threadlike mitochondria. Nuclei appear in the muscle buds, and gradually increase in number as the buds elongate into fibers. Most of them seem to come from the old fibers in the explant, as no nuclear division has been observed. This, together with the fact that the tips of the muscle buds are usually broad and flat and possessed of numerous amoeboid processes, while the main body of the fiber is often slender and devoid of processes, would indicate that amoeboid ends pull the fibers out of the explant and their nuclei along with them. Small fibers that are entirely isolated from the muscle plexus and the explant occasionally show rhythmical contractions without indication of any cross-striations (M. R. Lewis, 1915).

7. *Cartilage cells:*

When hyaline cartilage of the chick embryo is cultivated in plasma plus embryonic extract, the hyaline substance disappears and the small cartilage cells enlarge, become spindle-shaped, and grow into close contact with one another, forming thin membranes. With repeated transplantations these cells multiply by mitosis, and have been cultivated for over three months (Fischer, 1922*b*).

8. *Spleen cells:*

Cultures from the spleen of young chick embryos, seven to nine days' incubation, show profuse outgrowths of mesenchyme cells, frequent sheets of mesothelium, and some blood cells, mostly of the non-granular type, while those from older embryos show few or no migrating mesenchyme or mesothelial cells but many blood cells of various types. A few clasmatocytes are usually present from cultures of all ages. The mesenchyme cells show the usual reticular type of outgrowth. The amoeboid cells, lymphocytes, granulocytes, monocytes, and clasmatocytes always remain free, and show no tendency toward the formation of reticuli or membranes. Rioch (1923) observed, in addition to non-granular cells, two types of granulocytes, one containing round granules, the other spindle-shaped granules. They displayed striking amoeboid activity and rapid migration.

9. *Kidney-epithelial cells:*

The outgrowths from the collecting tubules, convoluted tubules, glomeruli, and undifferentiated nephrogenous tissue of the metanephros proceed in three different ways, according to Rienhoff (1922). He found that when the cut end of a collecting or convoluted tubule was at the margin of the explant, the kidney epithelium migrated quite as early and as rapidly as the mesenchymal tissue. Such outgrowths formed sheets or membranes that were spread out flat against the cover glass with no indication of tubular formation or further differentiation. Mitoses were common, and no apparent dedifferentiation occurred. In the event that a well-advanced marginal growth of mesenchyme had preceded that of the renal epithelium, so that there was some depth of cells into which the latter could grow, the collecting tubules grew out as such, often carrying undifferentiated nephrogenic tissue, which continued its differentiation and organization in the migratory zone in a fairly normal manner. When the marginal zone of mesenchyme was not thick, attempts at organization were abortive, although there was differentiation.

10. *Endodermal cells:*

Endoderm from the stomach and intestine of chick embryos migrates out as a continuous sheet or membrane with occasional detached cells at the periphery (Plate II *b* and *d*). As these cells spread out from the epithelial lining of the intestine on the undersurface of the cover glass, they tend to flatten out more and more, those at or near the periphery becoming exceedingly thin. The cells at the edge of the membrane usually show projecting processes of changing form. Within the membrane the adjoining edges of the cells are held together by a cement substance which is revealed by the silver nitrate method (Lewis and Lewis, 1912*b*). In the Locke-bouillon-dextrose medium, cell division is rare. The cells maintain their epithelial character for many days, and show no tendency toward further differentiation or dedifferentiation. In older cultures there is the usual accumulation of degeneration granules and vacuoles, the latter more marked in cells near the periphery. When cultures are unduly disturbed the membranes often contract toward the explant.

11. *Liver cells:*

The embryonic chick liver cells migrate out from the explant as a sheet or membrane one cell thick and similar to endodermal membranes. Lynch (1921) cultivated embryonic liver in a Locke-bouillon-dextrose medium (Plate II *a* and *c*). The best cultures were obtained from five- to twelve-day embryos. There was some migration from livers of thirteen- to sixteen-day embryos but none from the older ones. Do the liver cells lose their ability to migrate in this medium, or are substances developed in the liver which inhibit them, so that the cells apparently lose their migratory power before the time of hatching? With increased differentiation seems to go a loss of plasticity. Not all cultures, even from the younger embryos, show liver-cell migration; it is quite frequent, however, and usually begins within twenty-four hours, and occasionally as early as four hours, or it may not begin for forty-eight hours or longer. As the liver cells push out from the explant they show a more or less irregular border with one or two changing triangular processes. This changing, irregular border persists along the advancing edge of the membrane. Growths of the liver in Locke solution are often complicated by the formation of a large amount of fibrin threads and network. This is peculiar to liver cultures, and is probably due to the formation of fibrinogen by the liver cells. Levi (1922*b*) describes the division of the liver cells in a fifty-two-hour culture of the liver from a five-day embryo. These cells do not have the active blebs so common to other types of dividing cells in tissue cultures. They retain their characteristics through division and migration; they remain thick, polyhedral in shape, and full of small granules which seem to be mitochondria.

12. *Ectodermal cells:*

Embryonic ectoderm from various species of animals (Amphibia: Oppel, 1912*b;* Holmes, 1913*b*, 1914*a;* chick: Lambert, 1912*a;* Fischer, 1921*b*, 1922*a;* W. H. Lewis, 1923*e;* mammal: Drew, 1922) migrates as a sheet or membrane (Plate III). The cells at the edge of the membranes show active amoeboid processes (Plate III *d*), those within the membrane less so since they usually abut one another by rather clear-cut edges where a liberal amount of cement substance can be demonstrated with silver nitrate. The activity of the amoeboid processes at the edge of the membrane gives the impression that they are responsible for pulling out the membrane under considerable tension, as marked retraction of a part or the whole sheet toward the explant often occurs when the culture is disturbed. Although ectodermal cells have a marked adhesive affinity for one another and usually form continuous sheets without gaps, loose, almost reticular areas sometimes occur, and individual cells with numerous active pseudopodia not infrequently migrate entirely away from the other cells. The membranes vary from one to three cells in thickness. Near the explant the individual cells are usually much thicker than at the periphery, where they may become flattened out into extremely thin plates (Plate III *b*). In an ordinary plasma or fluid medium ectodermal cells show a limited amount of mitosis, and their ability to survive without subcultures is greater than that of most cells. Holmes was able to keep cultures of amphibian ectoderm alive for over three months by subcultures, and Fischer has carried pure cultures of embryonic chick ectoderm for a similar period by subcultures in plasma-embryonic juice medium, the cells continuing to proliferate and form new membranes without indications of dedifferentiation. If the culture medium was not renewed, degenerative changes appeared, i.e., breaking-up of the mitochondrial threads into rods and granules and the accumulation of numerous neutral-red granules and vacuoles. No enlarged centrospheres were observed during this process. The membranes also disintegrated. Some cells retracted into globular forms, others were pulled out into long, thin strands.

13. *Nerve fibers:*

The outgrowth of nerve fibers was first observed by Harrison (1907, 1911*a*), using explants of the embryonic neural tube and cranial ganglia of the frog in lymph. The free end of each fiber was enlarged and provided with fine processes or pseudopodia. This amoeboid end, as it moved away from the explant, appeared to draw the fiber out. The lengthening of the fiber varied from 15.6 micra to 56 micra per hour. Sometimes the nerve cells were so isolated from the mass of cells in the explant that the origin of a fiber from an individual cell could be seen. The longest fiber observed was 631 micra. Many anastomoses were seen between the fibers, some of which were later resolved. Harrison's experiments served to establish completely the concept that the nerve fiber is formed as the outgrowth of a single cell. One of the necessary conditions of the outgrowth of nerve fibers, just as with the migration of various types of cells, was some solid or semisolid support.

Burrows (1911) observed a similar outgrowth of nerve fibers from nerve cells in explants of the neural tube of chick embryos in chicken plasma. The greatest activity was in cultures of forty-eight to seventy-two hours. During this period the growth was from 1 to 1½ micra per minute, and the fibers sometimes attained a length of 1 to 2 millimeters. Burrows noted the characteristic amoeboid end showing the constant retraction and formation of pseudopodia.

The axis cylinders from brain cells of young chick embryos (six to ten days) grow out more readily than those from older or younger embryos, and those from the brains of cats six weeks old, rabbits two months old, and dogs three weeks old grow still more slowly than those from embryonic nervous tissue, and the percentage of outgrowths is very much less (Ingebrigtsen, 1913*b*).

The outgrowth of sympathetic nerve fibers (Plate V *c*) is similar to that of nerve fibers from the central nervous system (Lewis and Lewis, 1911*b*, 1912*a*). They have been observed to arise from the small sympathetic ganglia of the intestine, stomach, heart, liver, adrenal, and kidney of chick embryos of various ages. It has been found that these fibers grow out not only in plasma but in

Locke-bouillon-dextrose solution, in Locke solution, and even in simple sodium chloride solution. The sympathetic fibers are often extremely rich in very fine lateral branches or processes that extend and retract much as do those at the enlarged terminal tip.

After fixation the nerve fibers from both the central nervous system and the sympathetic system show the typical fibrillae. The most careful examination of the living fibers fails to reveal them, however (T. Matsumoto, 1920). We have repeatedly examined the living fibers with bright- and dark-field illumination without detecting neurofibrillae, and have come to the conclusion that these are probably due to the peculiar manner in which the apparently homogeneous cytoplasm coagulates. The free movement of the mitochondria and granules in the fibers, observed by Matsumoto and often by ourselves, tends to confirm the view that the cytoplasm of the nerve fibers is of a homogeneous semifluid nature. An appearance resembling neurofibrillae is often seen where several living fibers, closely adherent, run for some distance parallel with one another. In such places the adherent limiting membranes give a distinct striation, especially with the dark field.

When axis cylinders are severed from their origin they undergo degenerative changes that are not visible until after twenty hours. In the course of the next two days degeneration is complete. The central ends retract somewhat, but later some of them send out new axis cylinders (Ingebrigtsen, 1913*b*).

14. *Retinal cells:*

The pigmented mesenchymal cells of the embryonic choroid begin to migrate somewhat earlier than the pigmented epithelial cells, either singly or in anastomosing "syncytial" groups, according to Luna (1920). He made no especial effort to distinguish syncytial from adherent reticuli, however. The cells move slowly, and alter but little their original forms. Luna believes that they fuse not only with one another but also with ordinary mesenchyme cells in such a manner that the pigment granules can pass from the pigmented into the non-pigmented cells. His evidence is very meager, and he was evidently unaware of the fact, as shown by S. Matsumoto (1918*a*, *b*) and Smith (1921*b*), that loose pigment granules, which are common in such cultures, are readily ingested by the cells of the cornea and by the ordinary mesenchyme cells. Even more remarkable and more open to doubt is Luna's (1917) statement that the pigmented epithelium of the retina sometimes fuses or forms a syncytium with ordinary mesenchyme.

Cultures of pigment epithelium of the retina from the chick embryo have been carried on by Luna (1917) and Smith (1920*b*). They found that pigment epithelial cells migrate out from the small explants in thin sheets or membranes, rarely as a loose reticulum. Luna considers and figures these membranes as though they were syncytial in structure, while Smith's figures and our own observations lead us to believe that there is no actual fusion of the cells, merely adhesion. Individual cells occasionally migrate away from the membrane. When the cultures are first examined the rod-shaped pigment granules show but little activity; under the influence of light and heat, however, their movements become much accelerated. They tend to become clustered at one side of the nucleus, probably about the centriole. Various stages in the development of the pigment granules, from small, colorless rods, through different shades of gray to the deep black, were observed by Smith. He could find no evidence of a mitochondrial, nuclear, or fatty origin for these granules. They stain readily with neutral red, never with Janus green, and retain the red color even after fixation, thus differing from the ordinary neutral-red granules which accumulate in all types of cells. Smith's observations would seem to indicate that the pigment granules are direct products of the cytoplasm.

VII. CHARACTERISTICS OF MIGRATING ADULT CELLS

1. *Lymph-node cells:*

The lymphocytes are usually the first cells to migrate out from explants of human lymph nodes (Plate VIII), and appear within an hour after the cultures are made (Lewis and Webster, 1921*a*). The migrating lymphocytes are more or less elongated or finger-shaped, with the nucleus near the anterior end, and frequently small fluid pseudopods are present. When they come to rest, however, they become round and present the usual appearance of these cells. The maximum rate of migration of human lymphocytes in auto- or homoplasma is about 0.03 mm. per minute. Most of the migratory lymphocytes live only a few days, but with proper renewal of the medium they may continue to live for a long time without indication of any dedifferentiation.

In pure plasma cultures, according to Maximow (1923*a*, *b*), the lymphocytes show no clear progressive development; they migrate and multiply by mitosis, but ultimately die out after two or three subcultures. When bone-marrow extract is added to the medium the results are very different in part of the cultures. The migratory cells out in the clot die, but those in the explant and in the thicker region of the outgrowth near it divide actively, and may in some cases show very astonishing changes. Some of the small lymphocytes, according to Maximow, may become transformed into typical plasma cells, others into large lymphocytes, which in turn divide mitotically and suffer during this multiplication a differentiation in different directions. First arise leucoblasts or pseudomyelocytes, then myelocytes with few granules, then, under continued division, typical myelocytes rich in granules, and finally myelocytes with horseshoe-shaped nuclei. In some cases pseudo-eosinophile granular micromyelocytes arise directly from the small lymphocytes. Some of the small lymphocytes may enlarge, according to Maximow, into amoeboid phagocytic cells, like the dye-ingesting polyblasts which arise from the reticular cells. The increase may be at the expense of the large lymphocytes and the reverse process may also take place, especially in the loose part of the newly formed tissue. Unfortunately, Maximow's observations are based almost entirely on fixed, sectioned, and stained explants with their surrounding outgrowths. There is no clear indication that he has followed all these changes in living cells, and until this is done reasonable doubt will exist in the mind of the reader as to whether such transformations actually occur.

Several other types of cells migrate out from lymph nodes: a small, irregular, rather rapidly moving wandering cell, corresponding probably to the polyblasts of Maximow; and a large, slowly moving endothelioid-like cell (Lewis and Webster, 1921*b*, *c*), corresponding to the reticular cells of Maximow. From the latter arise giant cells by amitotic division of the nucleus (Lewis and Webster, 1921*b*) or by the fusion of several cells (Maximow, 1923*a*). The reticular cells, as well as the fibroblasts, according to Maximow, arise directly from the syncytial reticulum of the explant, and in plasma cultures with bone-marrow extract develop luxuriously in the explant, where they often reach gigantic dimensions, become filled with pigment and fat, and take on the character of epithelioid macrophages. Others migrate into the plasma clot, and remain for a long time as isolated amoeboid polyblasts, or multiply rapidly, decrease in size, lose their pigment, and may take on a fibroblastic appearance. In the ordinary plasma cultures of Lewis and Webster no transformations between the reticular cells (endothelial or endothelioid) and fibroblasts were noted. The fibroblasts that migrated out were always quite distinct, and resembled those from other tissues. The reticular or endothelioid cells are highly phagocytic, and often become engorged with dead lymphocytes.

2. *Blood cells:*

Carrel and Ebeling (1922*b*) cultivated the white blood cells of adult chickens in plasma plus embryonic extract for three months. The migration and proliferation was somewhat slower than that of fibroblasts. Under certain conditions the large mononuclears changed into fibroblast-like cells. The figures illustrating these cells appear to us to indicate changes in form rather than an actual change into true fibroblasts. We have often observed clasmatocytes elongate into fibroblast-like cells but never actually to change into fibroblasts. Such a transformation would be highly remarkable, and would need abundant evidence. Carrel and Ebeling (1922*e*) find that the serum from leukocytic cultures produces an increased activity of homologous fibroblasts, probably due, they suggest, to growth-promoting substances secreted by the leukocytes. The presence of a foreign proteid under certain conditions determines a more abundant leukocytic secretion.

The belief that blood cells can change into fibroblasts was expressed by Awrorow and Timofejewskij (1914). They claimed that in cultures of leukaemic blood mitotic multiplication of the lymphocytes, myeloblasts, neutrophiles, and eosinophiles occurs and that the myeloblasts and lymphocytes under certain conditions hypertrophy and form macrophages, giant cells, and spindle or multipolar branched cells resembling fibroblasts. This lends support, they believe, to the theory that such cells may take part in the regeneration of tissue in wound healing.

3. *Amoebocytes:*

Leo Loeb (1920, 1921*a*, *b*) has studied the migration and movements of amoebocytes from the blood of *Limulus*. Their amoeboid movement depends primarily, he believes, upon alternating changes in the consistency of the protoplasm, a phase of liquefaction being followed by a phase of hardening. He has subjected these cells to most careful analysis, observing the various forms of pseudopods under different conditions, the movements of the granuloplasm and the part each plays in the migration and spreading-out of these cells on solids.

4. *Bone-marrow cells:*

Adult chicken bone marrow has been cultivated in plasma by Foot (1912, 1913) and Erdmann (1917*a*, *b*), and that of the new-born rabbit by Maximow (1916*a*). In the normal bone marrow are to be found blood cells, eosinophiles, erythrocytes, myelocytes, microlymphocytes, macrolymphocytes, connective tissue, and fat cells. In Foot's (1913) paper he states that the small microlymphocytes, which migrate in considerable numbers, are transformed into macrolymphocytes, later into large, mononuclear forms, and then into myelocytes and polymorphonuclear leukocytes. These change into what he calls the cell-culture type. Not only does the cell-culture type originate from lymphocyte forms, but this "stem cell" can be transformed through the transition stage of amoeboid forms into "giant cells" and syncytia, as well as into the cell-culture type. Erdmann was unable to find any of these transition stages, and does not believe that Foot's observations are correct. She finds during the first period that the erythrocytes and nearly full-grown erythroblasts degenerate, the granulocytes ripen and later decay, the eosinophile mononuclear or polynuclear leukocytes, after rapid multiplication, lose their granules, flatten out, and form chains of acidophile character which undergo slow destruction. The myelocytes, at first amoeboid-like, behave as phagocytes; they enlarge, seldom divide, and their cytoplasm vacuolizes and disappears. The microlymphocytes show no signs of multiplying and later degenerate. The large, mononuclear lymphocytes migrate in numbers after the first day, form now and then fine granules, become vacuolized, and degenerate. The so-called *fat cells* flatten out, their large, fat globules divide into smaller droplets, their cytoplasm vacuolizes, needle-like projections appear, and they are finally transformed into fibroblast-like cells with vacuolated cytoplasm. Not all fat cells are transformed into fibroblasts or "Risenzellen," but some disintegrate. Elongated connective-tissue cells with very fine, pointed projections migrate, store fat droplets, and partially vacuolize. During the second period small and large basophile cells, with vesicular nuclei, and cells of intermediate type, between the "histotype Wanderzellen" (Danchakoff) and embryonic mesenchyme cell, migrate. They send out penetrating needle- and bristle-like projections, divide into phagocytes, lose their projections, and partially vacuolize, assuming the form of the "cell-culture" type. The "Risenzellen" of Foot comprehend several types: (1) transformed fat cells and elongated, vacuolized connective-tissue cells; (2) basophile cells of the bone-marrow network, related to the "histotype Wanderzelle" of Danchakoff; (3) some few myelocytes and flattened-out eosinophile mononuclear or polymorphonuclear leukocytes. Some types die out while others change character and live for long periods. After fourteen days the only surviving cells are: (1) the cell-culture type (modified fat cells and newly formed wandering cells of the mesenchyme-like type) and (2) elongated connective-tissue cells. They both belong to the connective-tissue type.

Maximow (1916*a*) observed on the first day, in cultures of the bone marrow of new-born rabbits, many granular and non-granular wandering cells. The granular cells, myelocytes, leukocytes of various types, and pseudo-eosinophiles lived for five days or longer, the myelocytes still dividing by mitosis. The majority of cells degenerated, and died without any backward differentiation. The non-granular amoeboid elements were exceedingly numerous and proliferated intensely. All transition forms, from small lymphocytes to large polyblasts of macrophagic character, were observed. The fibroblasts migrated and multiplied extensively by mitosis. By the fourth day they had formed a dense meshwork zone, 2 mm. wide, of branching cells. The wandering cells were dispersed in the interspaces between the fibroblasts.

5. *Epithelial cells:*

Adult epithelium usually migrates as a sheet or membrane of flattened cells. The pigmented epithelial cells of the retina and iris and the epithelial cells of the skin of the frog, according to Uhlenhuth (1916*a*), lose some of their characteristic structures, such as the cuticular cap and the basal membrane, in the change from the normal differential environment to the uniform environment of the culture. The form of the migrating cells from the skin of the adult frog varies, according to Uhlenhuth (1915), with the consistency of the medium. They are polyhedral in firm, fusiform or threadlike in soft, and round in liquid media. We are inclined to believe, however, that factors other than consistency of the medium determines the form, since embryonic epithelium may assume all the foregoing forms in a simple fluid medium.

6. *Connective-tissue cells:*

Adult connective-tissue fibroblasts migrate rather slowly in cultures of such organs as the lymph nodes, spleen, thymus, thyroid, and kidney, and form a loose reticulum of spindle cells with comparatively few processes. They do not have the plasticity or diversity of form seen in embryonic mesenchyme. The connective tissue from tumors behaves in cultures, according to Drew (1922), more like embryonic mesenchyme than like the adult tissue from which it is derived.

7. *Tumor cells:*

Both sarcoma and carcinoma cells from tumors of rats, mice, dogs, chickens, and man have been cultivated by Burrows, Burns and Suzuki (1917), Carrel and Burrows (1910*b*, *c;* 1911*b*, *f*), Champy (1921), Champy and Coca (1914*a*), Doyen, Lytchkowsky, and Smyrnoff (1913); Drew (1922, 1923*a*); Kimura (1919); Lambert (1912*d*, 1913*b*, 1916*b*), Lambert and Hanes (1911*a*, *b*, *c*, *d*, *e*, *g*), Losee and Ebeling (1914*b*), Maccabruni (1914), Ruffo (1917), Thomson and Thomson (1914), and Volpino (1910) (Plate V *d*). The sarcoma cells tend to migrate like the connective-tissue cells, and either form reticuli or remain isolated, while the carcinoma cells migrate in sheets or membranes like the epithelium. Chicken sarcoma cells migrated very extensively in the plasma of the individual bearing the tumor; the latent period was less and there was more amoeboid activity and more rapid cell division than with connective tissue. When sarcoma was cultivated in the plasma of a normal animal the growth was less extensive than in autoplasma, and when the plasma of another sarcomatous animal was used there was little or no outgrowth. The plasma of sarcomatous animals acquires, then, the property of inhibiting the growth of a sarcoma taken from another animal (Carrel and Burrows, 1911*a*). Mouse sarcoma cells, which migrated readily in the plasma of normal rats, showed little or no activity in the plasma of rats immunized by mouse sarcoma injections (Lambert and Hanes, 1911*b*), but the cytotoxins thus produced were not specific for the tissue injected. Lambert (1913*b*) found that sarcoma cells, although more active in primary cultures than connective tissue, became less and less so when propagated over a long period of time in subcultures.

Drew (1922) obtained in cultures of a mammary carcinoma of the mouse at first only rapid growth of the stroma. In forty-eight hours the parenchyma began to push through the stroma in the form of finger-shaped masses. If an abundant outgrowth of both connective-tissue stroma and parenchyma occurred, the latter showed an acinous type of structure in the later subcultures as growth progressed. If the carcinoma cells were free from stroma there was no tendency to form acini.

VIII. STRUCTURE OF NORMAL TISSUE-CULTURE CELLS

1. *Cytoplasm:*

The thin, flat embryonic mesenchyme cells that are adherent to the cover-glass in young cultures in the Locke-bouillon-dextrose medium offer very favorable objects for examination and experimentation. The cytoplasm of such cells is optically structureless, and appears homogeneous with both bright-

and dark-field illumination. The networks and granules that appear on fixation or death are coagulation products, and do not represent in any sense structures that are present in the living cytoplasm. There is no visible cell membrane and no sharp distinction between ectoplasm and endoplasm. In the cytoplasm are imbedded thread- and rod-shaped mitochondria, which slowly change position, and varying numbers of small granules, with a marked affinity for neutral red, which often move rapidly back and forth between the center and periphery. Fat globules may or may not be present. The centrioles are not distinguishable in the living cell. The migration, flattening out, and general plasticity of cells and the movements of the mitochondria and of the granules indicate that the cytoplasm is semifluid. The movements of the granules would seem to indicate the existence of currents of more fluid cytoplasm or alternating local changes in consistency in the depths of the cytoplasm, just as the formation of pseudopodia indicates similar alternating changes in consistency at the surface. Under the ordinary conditions of plasma cultures the cytoplasm also varies in consistency; according to Levi (1918*a*), it is sometimes more fluid, sometimes more solid. With ordinary light, Brownian motion of the small granules is not usually seen; with the dark field, however, it is often observed. This difference may be due to the changed conditions, as for dark-field illumination the cultures must be sealed on a flat slide without an air space. Under such conditions oxygen is excluded and the cells die in an hour or two (W. H. Lewis, 1923*c*). Changes in consistency occur during mitosis, as evidenced by a rounding-up of the cells during prophase and early metaphase.

The fluidity of the cytoplasm can be *increased* by ether (Cash, 1919), hypotonic solutions (Hogue, 1919), and alkalies (Loeb and Blanchard, 1922; M. R. Lewis, 1923) without killing the cell. Potassium cyanide N/10 or N/20 dissolves the cytoplasm (Olivo, 1922). This is probably not due to the direct action of the cyanide but to the alkaline solution produced by it, as strong alkaline solutions kill the cell and dissolve the cytoplasm. The granules in rounded-up degenerating cells exhibit more Brownian movement than in normal cells.

The fluidity of the cytoplasm is *decreased*, and coagulation (granulation) appears when acid (sulphuric, nitric, hydrochloric, acetic, lactic, citric, formic, oxalic, or picric) is added to the medium to produce a H-ion concentration of 4.4. All movements of the cells stop, the mitochondria and the granules remain quiet, and unless the solution is washed off in a few seconds, before the mitochondria change to vesicles, the cells will die. If the solution is washed off the cytoplasm becomes homogeneous and the mitochondria and neutral-red bodies return to normal. With a solution of pH 4.6 the cytoplasm remains homogeneous and the mitochondria and granules become less active, indicating a decrease in fluidity. If the solution is soon washed off, the mitochondria and granules become active again and the cells live and grow about as well as the controls. Such reversible gelation changes can be repeated several times on the same cells (M. R. Lewis, 1923). The stiffening of the cytoplasm of the amoebocytes of *Limulus* by acids has been observed by Loeb and Blanchard (1922). Strong solutions of dextrose (3–5 per cent) and certain bacteria increase the consistency of the cytoplasm of degenerating cells.

2. *Nucleus:*

The living nucleus is optically structureless and homogeneous with both bright- and dark-field illumination. It has a slightly different refractive index from that of the cytoplasm and is slightly more opaque. No nuclear membrane is visible but with the dark field a thin, white line borders it. No linin thread or chromatin granules are to be seen. These are fixation, coagulation, or precipitation products, and do not represent living structures. The nucleoli, one or two in each nucleus, are irregular in outline and change form and position. With the dark field they appear granular.

The consistency of the nucleus can be increased by acids and decreased by alkalies. If sufficient acid (sulphuric, nitric, hydrochloric, acetic, lactic, citric, formic, oxalic, or picric) is added to the medium to produce a H-ion concentration of 4.6–3.8, and the culture is bathed once or twice with this freshly made solution, very minute granules immediately begin to appear in the nucleus, a nuclear membrane becomes visible, and the nucleoli become brighter and clearer. This is followed by continued gelation of the nuclear substance into larger and larger granules, and a broadening and brightening of its limiting membrane. Within a few seconds the nucleus becomes slightly shrunken and distorted into a coarse reticulum with here and there larger masses inclosed in a thick, shiny membrane. If the acid solution is soon washed off the nucleus returns to the homogeneous state almost immediately after the pH 4.6 solutions, more slowly after the pH 4.4–3.8 solutions. Gelation of the nucleus can be brought about and reversed several times in succession in the same cell, but these cells, although they multiply, do not live as long as the controls or as cells that have been gelated only once.

When cells are bathed with solutions of pH 9–9.6 the nucleus becomes dissolved and loses its membrane. More alkaline solutions (pH 10) are usually necessary to dissolve the nucleolus. This effect may be produced, not only by means of ammonia vapor, ammonium hydrate, and ammonium carbonate, but also by sodium bicarbonate, sodium hydroxide, potassium hydroxide, and lithium carbonate. If the nucleous is dissolved the cells often remain rounded-up, even many hours after the alkali is removed. Cells seldom recover after the nucleolus is dissolved, and when the alkali is washed off, although the nucleoli again appear, the nuclear material, instead of returning to a homogeneous state, usually becomes somewhat granular. The amount of acid necessary to produce an immediately perceptible change in the state of the cell and also the amount of alkali necessary to dissolve the nucleolus is greater than that in which these cells can live, as has been shown by Lewis and Felton (1921).

3. *Mitochondria:*

The mitochondria are easily observed in the living cells. They stain beautifully with Janus green and Janus black 2b, but since these dyes injure the cell and produce mitochondrial distortions, observations that are to continue for more than fifteen minutes should be made without them. Neutral red and brilliant cresyl blue do not stain the mitochondria. Methylene blue sometimes gives them a very transitory blue color. Janus green stains the mitochondria rather slowly, several minutes (three to ten) often being required to get the deep blue stain. In Janus green acid (pH 4.4), Janus green–pyrogallic acid (pH 6.4), and Janus green–potassium cyanide (pH 8.6) media, the mitochondria fail to stain, but if replaced with a fresh Locke-bouillon-dextrose medium without the dye, the mitochondria are immediately colored. This would seem to indicate that the dye, reduced to a leucobase, was present in the mitochondria and became reoxidized when fresh normal medium was added. If, after the mitochondria are deeply stained with Janus green, the medium containing the dye is replaced by an acid one (Locke-bouillon-dextrose medium), the dye remains in the mitochondria. If replaced by a medium containing potassium cyanide (pH 8.6), the color fades rapidly. If the cyanide-containing medium is replaced by a normal medium without the dye, the color returns immediately. This can be repeated many times on the same cell with the same result. Since no new dye enters, it is quite evident that the dye undergoes alternating changes of reduction and oxidation (M. R. Lewis, 1923).

In the healthy cells the mitochondria are in the form of threads, rods, and granules, the wavy threads predominating. Slight alterations in the condition of the cell or culture seem to produce marked variations in the form, from rich branching and anastomosing plexuses of long threads to rods, short rods, and granules. The diameter varies likewise, though as a rule it is fairly uniform. In the older cultures, where the cells are degenerating, the threads become beaded and segmented into short rods and granules. The latter may become extremely small, reaching the limits of visibility; sometimes, however, the rod and granular forms swell into vesicles of various sizes. During mitosis the threads usually change into short rods; the method of the return to threads after division has not been followed.

When acid medium pH 4.4 is used, the mitochondria become varicose and may swell into vesicles. If the acid solution is washed off before they change into vesicles, they recover their usual filamentous form. Weak solutions of potassium permanganate produce similar forms, but the reversibility has not been tested. Similar forms appear with alcohol, ether, and poor fixation. When the cells are bathed with an alkaline solution pH 9.6, the mitochondria immediately shorten without segmentation into thick rods and granules.

The mitochondria vary in number in the healthy cells of young cultures from many to as few as ten or fifteen. As the cultures get older the average number per cell often decreases. The number can be very markedly decreased by carbolic acid. As a result many cells die, but in those which survive most of the mitochondria slowly fade away until, after twenty-four hours, some cells may contain only one or two long rods or filaments. The number seems to increase in some of the more favorable cultures without any change in the medium. With the addition of a slight amount of acetone to the medium the number sometimes increases.

The mitochondria are continually twisting and turning, bending into curves and loops. When the cells are paralyzed by such substances as acids, illuminating gas, and potassium cyanide, they become quiet. If the acid medium is replaced by fresh medium their activity is often increased above normal. Such changes in activity are probably secondary to changes in the cytoplasmic consistency.

4. *Granules and vacuoles:*

The normal mesenchyme cells contain very few or no granules. Under the abnormal conditions in cultures the granules increase in number and vacuoles form about them. The latter develop rapidly, and are more numerous in cultures with simple Locke solution than with plasma or Locke-bouillon-dextrose medium. The importance of dextrose in preventing their formation and in bringing about the return of vacuolated cells to a more normal non-vacuolated condition has been shown by M. R. Lewis (1922). The introduction of typhoid bacilli (M. R. Lewis, 1920*c*) and of ammonia vapor causes a very rapid formation of vacuoles (Plate IX *c*). Since such highly granular and vacuolated cells die off more rapidly than the non-vacuolated ones, the granules and vacuoles have been called *degeneration* granules and vacuoles. As they have a marked affinity for neutral red they are often called *neutral-red* bodies. Similar vacuoles with an affinity for neutral red often develop about ingested particles. Such digestive vacuoles and those formed during degeneration and starvation by autolysis or self-digestion of the cytoplasm have much in common. In many cultures the latter predominate almost exclusively and are the type with which we are especially concerned here and in the section on cell degeneration.

The vacuoles are sometimes drawn out into long channels, especially when stained with brilliant cresyl blue. The channels are very unstable, anastomosing or changing back again into spherical vacuoles. If a medium containing a dilute solution of urea is used on the culture and a minute fragment of urease tablet added, the vacuoles change into channels which extend far out into the cytoplasm, as the urease breaks down the urea. If previously stained with neutral red the color disappears. If the urea-urease solution is replaced by Locke-bouillon-dextrose solution the channels return to spherical vacuoles and the color partly returns.

These granules and vacuoles have a marked affinity not only for neutral red but also for methylene blue and brilliant cresyl blue. When stained with neutral red their color may vary somewhat, according to the condition of the culture, from pink (acid) or brick-red (neutral) in healthy cultures to a yellowish (alkaline) shade in the older, degenerate ones. The color is not easily changed by washing the culture with a weak acid or a weak alkaline medium. A rosy pink color can, however, be produced with a carbon dioxide medium pH 5.2 and a deep yellow with ammonia vapor. With strong solutions of sodium hydroxide and sodium bicarbonate (pH 9) they become yellow. With lactic and acetic acid in the medium (pH 5.2) they become paler, but any change in color is doubtful.

The vacuoles fail to stain in an acid (pH 5 or 4.8), neutral-red medium. If, after a culture is bathed for a few seconds in such a medium, the latter is replaced by a dye-free medium (Locke-bouillon-dextrose), the neutral red appears at once in the vacuoles and granules, indicating that the dye was probably present there as a colorless compound. If such cultures are washed in an acid medium the color disappears from the vacuolar fluid but remains in the granules. The same failure of the vacuoles to show color occurs in a potassium cyanide (pH 9.6) neutral-red medium. If this is washed off with a dye-free medium the red color appears immediately. Unlike the acid, the washing of a stained culture with a potassium cyanide (pH 9.6) medium causes the red color of the granules to disappear, but it returns when the cyanide is washed off. It requires a somewhat stronger solution of potassium cyanide to bring about the loss of color in the granules and vacuoles than in the mitochondria.

5. *Fat globules:*

Many of the connective-tissue cells in tissue cultures in Locke-bouillon-dextrose solution are entirely devoid of fat globules, others may contain a few or many. These bodies move slowly, their change in position being more frequently attendant upon the movement of the cell itself than upon currents in the cytoplasm. In abnormal cells the fat globules often begin to exhibit Brownian movement earlier than other cell granules. They seldom change shape but may increase in size and number in any given cell or in all the cells of a culture. The number of fat globules in these cells depends upon the fat present in the embryo and also upon that in the medium (Lambert, 1914*b*). All the cells of a culture become full of fat globules if a drop of yolk is added to the medium (M. R. Lewis, 1918). Foot (1912) claims that fat is actually ingested by the cells, while M. R. Lewis found that yolk stained with Sudan III appeared first as very minute granules which later grew in size. Krontowsky and Poleff (1914) claim to have observed an actual metamorphosis of fat. These authors think that it is neutral fat, while Champy (1920) holds that certain vacuoles found in plasma cultures are "graisse phosphorée." Fici (1921) made a number of observations upon the nature of the fat formed in tissue cultures. It is extremely difficult for us to draw any conclusions in regard to the fatty nature of bodies found in the living cell. As we have shown (Lewis and Lewis, 1915), fat globules do not stain with Nile blue in the living cell but do become stained in fixed cells, while vacuoles do stain with Nile blue in living cells but not in fixed cells. Mitochondria stain with Janus green in the living cells and fat remains unstained, but if a culture stained with Janus green is placed over osmic vapor the blue color leaves the mitochondria and accumulates in the fat globules. Fat globules in the living cells take up Sudan III after it has been in the medium for a number of hours, becoming a deep straw color rather than the bright red of fixed preparations. Fat globules become browned by osmic vapor and the mitochondria do not. Neither by means of Sudan III nor by osmic vapor was it possible to detect any formation or storage of fat in the mitochondria. Iodine fumes, which are a most effective fixing agent for the mitochondria, neutral-red bodies, and the cell in general, fail to preserve the fat globules, which, instead of remaining round, spread out into various queer shapes. When cultures or pieces of tissue have died under acid conditions without fixation, the most bizarre forms of fat bodies (which stain with Sudan III in alcohol) often appear, such as twists and curls, frequently lying outside of the cell body as though squeezed out.

IX. CELL DIVISION

1. *Mitosis:*

Mitotic division of the migratory cells occurs abundantly in suitable media, such as lymph or plasma or their various modifications, Locke-bouillon-dextrose medium, saline mixtures plus embryonic extract, etc. In primary cultures mitoses often occur during the first day, and may continue for days until the cells show considerable degeneration. Where cultures are carried on for weeks and months by subcultures, the increase in the number of cells is brought about principally by mitotic cell division. In cultures of connective tissue, mitoses are as frequent after ten years of subculturing as they were years before. The changes in form which the mesenchyme cells undergo during mitosis, as described by Levi (1916*d*) for plasma cultures, have been noted in abundance by us in Locke-bouillon-dextrose cultures. During the prophase and early metaphase stages the protoplasmic processes are gradually withdrawn into the body of the cell, which tends to assume an oval form. The cytoplasm becomes more refractive, less transparent, and probably firmer in consistency, and the threadlike mitochondria become short rods and granules.

The chromosomes, which become visible during prophase, have characteristic forms, such as bent loops, rods, and granules, from the beginning of visibility. Their arrangement into the median plate of the metaphase stage and subsequent migration to the poles can be followed with ease. After the chromosomal plates have moved to the poles and indentation has begun, numerous blunt, bleblike, actively changing pseudopodia appear, coming and going constantly during the few minutes of telephase. After division is completed, the daughter-cells enlarge and spread out, the nuclear masses round up and slowly enlarge, and elongated mitochondria again appear by an apparent increase in the mitochondrial substance. There does not seem to be an exactly equal distribution of the mitochondria to the two daughter-cells. Not all cells round up during division, and the mitochondria may remain more or less elongated.

The homogeneous *spindle area* appears to come from the nuclear sap, and toward the end of prophase the clear-cut nuclear outline is lost; but even at this stage a nuclear membrane reappears if weak acids are applied (M.R. Lewis, 1923). No traces of spindle fibers are to be seen at any stage of mitosis in the living cells under the usual conditions. If, however, acid media (pH 4.6) is added, typical spindle fibers become visible. They disappear again if the acid solution is replaced by a neutral one. This reversible gelation of the spindle can be repeated several times without killing the cell. Division stops abruptly during the period of gelation and, if the acid is not washed off, the cells soon die without further progress of the mitosis. When, however, the acid is washed off and the spindle fibers disappear, mitosis proceeds to completion and the cells may survive almost as long as controls (M.R. Lewis, 1923). These observations indicate that the spindle fibers are fixation artifacts. The duration of the various phases of mitosis have been recorded by Lambert (1914*b*), Levi (1916*d*), and Lewis and Lewis (1917*c*) for the connective-tissue cells. In comparing the observations of Levi on embryonic chick mesenchyme in plasma with our observations on similar cells in Locke-bouillon-dextrose solution, there will be found a fair agreement. We have allotted more time to the prophase—thirty to sixty minutes, as compared with five to twenty minutes by Levi. The estimates of Levi are for metaphase eight to thirteen minutes, anaphase three to seven minutes, and telephase two and one-half to six minutes. Ours are for metaphase two to twelve minutes, anaphase two to three minutes, telaphase three to twelve minutes, and the reconstruction period thirty to one hundred and twenty minutes. During the latter period the daughter-nuclei gradually increase in size to that of the resting stage. In fixed specimens the daughter-nuclei stain deeply and are easily distinguished from the paler resting nuclei. From the present data two or three hours would be a fair estimate of the total time involved from the beginning of prophase to the resting stage. Strangeways (1922) finds that the time from the beginning of one division to the beginning of the next by one of the daughter-cells was from eleven to twelve hours for embryonic chick cells in plasma plus embryonic extract.

2. *Atypical mitosis:*

Atypical mitoses in the migratory cells are rather rare. Lambert (1913*b*) observed mitotic division of the nucleus without division of the cytoplasm and multipolar mitoses in cultures of tumor cells. During the past ten years we have followed in living cells from embryonic chicks a few atypical mitoses, such as mitotic division of the nucleus without division of the cytoplasm and cells with two, three, four, or more spindles. The cells with two spindles evidently arose from binucleate cells. In almost every case two of the poles were close together, the other two some distance apart, and as the chromosomes passed to the poles three nuclei were formed, two of normal size and one double-sized one, resulting, when the cytoplasmic division was completed, in a binucleated cell with ordinary nuclei and a second cell with a giant nucleus. Very rarely a large cell with a single spindle containing about double the normal number of chromosomes has been encountered dividing into two large cells with giant nuclei. Coupling the preceding case with the last suggests one mode of origin of hyperchromatosis, so common in tumor cells.

3. *Amitosis:*

Many statements to the effect that amitosis occurs in tissue cultures are found in the literature. They are based, as a rule, not on actual observation of the complete cleavage of the nucleus but on the occurrence of the various stages seen in different nuclei, especially in fixed material. Lambert (1913*b*) made many attempts to follow direct cleavage of the nucleus in living cells without finding a single instance. We also have made many attempts on embryonic chick cells but have never succeeded. One may observe partial or almost complete cleavage followed by a return to the usual oval type. Holmes (1914*a*) noticed amitosis without cytoplasmic division in the ectodermal cells of adult frog that had been cultivated for some time with unchanged medium (blood serum and gelatin). He considered this phenomenon as indicating declining activity. Kreibisch (1914) describes the direct division of epithelial cells from the cornea and skin of the pig. The fourteen- to twenty-four-hour cultures were the best. Physiological types with equal daughter-cells and pathological types with unequal daughter-cells occurred. Lewis and Webster (1921*b*), after prolonged efforts, observed a single case of direct cleavage of the nucleus without division of the cytoplasm in an epithelioid giant cell. Lynch (1921) observed the direct division of the nucleus of an embryonic chick liver cell without division of the cytoplasm. There are so many indications of the process, however, especially in older cultures, that it is hard to believe it does not occur more frequently than the observations on living cells would seem to indicate.

4. *Nuclear fragmentation and budding:*

Various stages of nuclear fragmentation and budding are to be found in many of the older cultures, where other degenerative changes are evident. They are especially apparent in the fixed material. Nuclear fragmentation usually leads to unequal division of the nucleus into two to four or six parts, whose total bulk may not exceed that of a large resting nucleus. Macklin (1916*a*, *b*) noted that the mesenchyme cells of the older cultures often contained lobulated and fragmented nuclei with the fragments varying in size and shape and often without a nucleolus. Endothelial cells from the embryonic liver frequently exhibit similar nuclei in the older cultures (W. H. Lewis, 1922*b*). Such cells show very distinct signs of degeneration, extensive vacuolization, granular instead of threadlike mitochondria, and a reduction in the total mitochondrial content.

5. *Binucleate cells:*

Binucleate cells may arise by either mitotic or amitotic division of the nucleus without division of the cytoplasm. They occur in most of the various cell types found in cultures of both embryonic and adult tissues, such as the adult ectoderm of the frog (Holmes, 1914*a*), embryonic mesenchyme (Macklin, 1916, 1917), liver cells (Lynch, 1921), endothelium (W. H. Lewis, 1922*b*), and the wandering endothelioid cells from adult human lymph nodes (Lewis and Webster, 1921*b*). We have also noted them in embryonic ectoderm, endoderm, smooth muscle, heart muscle, kidney epithelium, and clasmatocytes. The binucleate cells tend to increase in number with the age of the culture, indicating that the accumulative injurious effects of the abnormal environment are in part, at least, responsible for their formation. Some binucleate cells contain one small centrosphere, some two, which suggests that the former may arise by amitotic, the latter by mitotic division of the nucleus. The nuclei of binucleate cells may undergo mitotic division. In some cases, as observed by Macklin, the chromatin material from the two nuclei become merged into one equitorial plate of chromosomes, and division proceeds as in mononuclear cells, while in others two equitorial plates of chromosomes are formed, with two spindles and four polar bodies. The former probably arise from the amitotic binucleate cells with one pair of centrioles, the latter from the mitotic binucleate cells with two pairs of centrioles.

6. *Giant cells:*

Giant cells with three to one hundred nuclei were observed by Lambert and Hanes (1911*g*) in cultures of rat spleen and rat bone marrow. They were especially numerous when human plasma was used as the culture medium. These cells moved actively and were highly phagocytic for dead cells and foreign bodies. They tended to spread out widely on the cover glass. Cultures of chicken spleen and intestine frequently produced similar giant cells. Lambert (1912*a*, *c*) also observed the formation of large foreign-body giant cells in cultures of the spleen from chicks of twenty days' incubation and newly hatched when lycopodium spores were introduced into the plasma medium. The large mononuclear wandering cells, pulp cells, and probably endothelioid cells, collected about the spores after the third day of cultivation, and fused to form the giant cells. Since the leukocytes remain active for only two or three days, these probably took no part in the formation of the giant cells. The connective-tissue cells likewise did not appear to take part in their formation. Connective-tissue cells from cultures of the heart showed no tendency to surround the spores or to form giant cells. The cover glass may act occasionally in spleen cultures as a foreign body, bringing about the formation of a giant cell. The number of nuclei in these cells may vary from three to one hundred (Lambert, 1912). Such cover-glass foreign-body giant cells are often seen in tumor cultures, especially mouse tumor in human plasma (Lambert, 1912). Lambert was not able to determine whether such cover-glass giant cells arise from a single cell or by fusion of cells. We have occasionally noted giant cells in cultures of the heart and also of the intestine of the chick embryo in fluid media. They appeared to come from the mesenchyme cells, not by fusion, but by division of the nucleus, probably by amitosis without division of the cytoplasm. Large phagocytic giant cells are of frequent occurrence in cultures of human lymph nodes in plasma (Lewis and Webster, 1921*b;* Maximow, 1923*a*). These giant cells resemble very closely those seen in ordinary sections of tuberculous lymph nodes. They possess a large central area which stains deeply with neutral red in the living cell, indicating that it contains much dead material; around this area the nuclei are arranged in the typical horseshoe fashion. These giant cells with two or many nuclei are identical in structure with the large mononuclear endothelioid wandering cells (reticular cells of Maximow), except that the nuclei are smaller. In cells with one or few nuclei there are to be found all stages of amitosis, and since we were able actually to observe a complete amitotic division of a nucleus, we are inclined to believe that these giant cells arise by amitotic division of the nucleus without division of the cytoplasm. Maximow, on the other hand, believes that these foreign-body giant cells arise by confluence of the reticular cells. His evidence, however, is not satisfactory.

X. POTENTIAL IMMORTALITY AND MULTIPLICATION OF SOMATIC CELLS

Although various types of cells migrate and divide in ordinary cultures with lymph, plasma, and saline media, with their various modifications, they ultimately die out, even when fresh medium is supplied or the tissue is divided and transferred to a fresh drop. The desirability of cultivating tissues indefinitely was early recognized, and to Carrel (1913*b*) and Ebeling (1913) is due the credit of finding a method. Their results indicate that embryonic fibroblasts can be cultivated indefinitely, and are thus potentially immortal when the necessary ingredient—embryonic extract or juice—is added to the plasma in varying proportions (1:2, etc). Subcultures into a fresh medium must be made every few days, usually every forty-eight hours. According to Carrel and Ebeling (1921*a*), the temporary multiplication of fibroblasts cultivated in adult plasma is due to the presence of small amounts of embryonic juice within the explant, and the indefinite multiplication of fibroblasts in a medium composed of plasma and embryonic juice is due to the latter ingredient. These authors have cultivated a strain of fibroblasts from the heart of a chick embryo for over ten years by repeated subcultures. Each fragment doubles itself in about forty-eight hours and is then divided, washed, and a fresh culture made with each piece. The growth (migration and multiplication) was as rapid after ten years as it was at first and many mitotic figures were seen. The general character of the culture and of the cells had not altered during this period, and the cells remained typical embryonic fibroblasts. Eighteen hundred and sixty generations of subcultures with 30,000 cultures had been reached when Ebeling published his account in 1922 (*a*).

The indefinite multiplication of fibroblasts in a medium composed of adult plasma and embryonic juice is due neither to the serum nor to the fibrin, but depends entirely upon substances contained in the embryonic juice. The embryonic juice does not give to the cells the power of using the constituents of the plasma. The rate of growth of the fibroblasts was found to be a function of concentration of the embryonic juice in the medium, so that it seems evident that the material employed by the fibroblasts in their indefinite multiplication *in vitro* was supplied by the embryonic juice (Carrel and Ebeling, 1921*a*, 1923). Ebeling (1921*b*) found in the cultivation of the nine-year-old strain of embryonic chick fibroblasts that a medium consisting of fibrinogen 12.5 per cent, chicken serum 37.5 per cent, and embryonic juice 50 per cent answered about as well for several generations as the usual plasma-embryonic juice medium.

According to Drew (1922, 1923*a*), continuous growth of normal cells and tumor cells by subcultures can be obtained by substituting for the plasma-embryonic extract medium a saline embryonic extract medium in which the calcium is present in the colloidal state. The substances in the embryonic juice which are necessary for continued growth will pass through a Berkefeld filter. He states that adult tissues and tumors appear to contain in lower concentration those substances which are necessary for the continued life of cells *in vitro*. The autolyzed extracts of normal adult tissues contain a growth-activating substance which, when added to cultures of normal adult cells, causes rapid proliferation. Extracts of tumors, prepared in the cold and not subjected to autolysis, also contain this substance; the tumor cells appear to be able to form it continuously.

Carrel (1913*b*) found that when a strain of connective tissue had been cultivated for a certain length of time by subcultures, a definite relation was established between the rate of growth of the cells and the composition of the medium. It was possible, by adding certain substances, such as embryonic juice, to foresee the extent to which a piece of tissue would increase in a given time. The rate of growth could be accelerated or retarded. The rate of multiplication varied inversely with the age of the animals from which the plasma was taken, but the decrease in the rate of multiplication was relatively greater than the increase in age, and was due to the increase of an inhibiting factor (Carrel and Ebeling, 1921*b*). The inhibiting action of homologous serum on the proliferation of fibroblasts, as compared with normal plasma, was increased after the serum had been heated to 56°–70° C. and decreased after it had been heated to 100° C. (Carrel and Ebeling, 1922*b*). The partial or complete inactivation of serum by shaking brought about a marked decrease in the activity of homologous fibroblasts (Carrel and Ebeling, 1922*c*).

An increase in the osmotic pressure of the plasma-embryonic juice medium by adding two parts of a 2 per cent sodium chloride solution or a decrease by adding two parts of distilled water at first stimulated cell proliferation, but eventually retarded it and proved to be unfavorable to growth in repeated subcultures of embryonic connective tissue. Dilution with two parts of Ringer's solution caused more extensive migration but no increase of proliferation over that seen in the normal control cultures. Cultures of connective tissue which had been growing under unfavorable conditions, due to changing the osmotic tension of the medium in which they had been cultivated, were revived by repeated subcultures in a plasma-embryonic extract medium (Ebeling, 1914).

It is probable that other somatic cells possess the power of indefinite proliferation. Fischer (1921*b*) cultivated ectodermal epithelium from the chick embryo in pure cultures by subcultures in the plasma-embryonic juice medium for over three months. The cells continued to proliferate by mitosis, and grew as pavement epithelial membranes without further differentiation.

XI. DIFFERENTIATION AND DEDIFFERENTIATION

1. *Maintenance of type:*

We have already noted that the various types of cells migrate each in its own characteristic manner, and that they retain their peculiarities in the primary cultures for many days and sometimes for weeks, or until the cells die. Mesenchyme, smooth muscle, heart muscle, skeletal muscle, ectoderm, endoderm, kidney epithelium, lymphocytes, blood cells, clasmatocytes, etc., retain their distinctive peculiarities, and can always be recognized as such in primary cultures until they die off. Mesenchyme or fibroblast cells, even after ten years in subcultures, cannot be distinguished from those in earlier cultures by the nature of the growth or the appearance of the cells (Ebeling, 1922*a*, *b*). Ectodermal epithelium of the chick embryo, after repeated subcultures for a period of over three months, still retained its epithelial character, forming flat sheets or membranes (Fischer, 1922*a*). Drew (1922) found that the heart muscle of chick embryos could be grown, generation after generation, in subcultures. All traces of the original explant disappeared, and the growth consisted of a thin, rapidly pulsating sheet of cells. This maintenance of cell type is often accompanied by mitotic cell division. Maintenance of cell types occurs in cultures of only one type (pure cell-type cultures) and in mixed cultures, where the mesenchyme does not unduly overgrow the other cell types. When the mesenchyme is abundant, epithelial cells may show progressive differentiation.

2. *Transformation without differentiation:*

There are certain transformations in the form and character of cells in cultures that are probably neither differentiation nor dedifferentiation, but merely adaptations to the abnormal environment. The transformation of adult endothelium into fibroblast-like cells, as observed by Maximow (1922) and others, or of embryonic mesenchyme into mesothelium and mesothelium in turn into multipolar mesenchyme cells (W. H. Lewis, 1923*a*), does not indicate either differentiation or dedifferentiation in the ordinary sense. The transformation of the reticular cells (endothelioid cells) of Maximow (1922, 1923*a*) in cultures of adult lymph nodes into large, polyblast-like amoeboid cells, into phagocytic macrophages, or into giant cells (Lewis and Webster, 1921*b*) is likewise probably not a real differentiation. So, too, the transformation of small lymphocytes into large ones and the large lymphocytes into small ones indicates, according to Maximow (1923*a*), that the small, medium, and large lymphocytes belong to a single-cell type. The transformation of the large mononuclear blood cells into fibroblast-like cells, described by Carrel and Ebeling (1922*d*), in pure cultures of adult chicken blood that were kept alive for over three months by subcultures in plasma plus embryonic extract is probably a transformation in form rather than a differentiation into another cell type. The clasmatocytes in our cultures sometimes assume such fibroblast-like forms but have never actually changed into real fibroblasts. Under certain conditions ectodermal cells may become spindle-shaped (Uhlenhuth, 1915), but such alterations in form are not dependent upon a differentiation or dedifferentiation of the cells.

3. *Differentiation in migratory zone:*

In submitting the evidence for differentiation in cultures it will be best to consider first the changes in the migratory cells and their descendants, and secondly, the cells that remain in the explant. Holmes (1914*a*) states that the epithelial outgrowths from very young amphibian embryos showed changes comparable to those taking place in the epithelium on the body of the larva. There was a progressive loss of yolk spherules, and as the yolk disappeared the cells became thinner and thinner. From cells originally alike in appearance there came to be a differentiation into cells of the vacuolar type, granular cells (rare), ciliated cells, and cells of typical pavement epithelium. His cultures were kept alive for several months by the occasional transfer into a fresh culture medium and mitotic cell divisions were noted fifty days after explantation. Pure cultures of embryonic chick ectoderm do not differentiate, even after two or three months of subcultures, but when connective tissue is added to such cultures, Ebeling and Fischer (1922) found after several subcultures that the fibroblasts overgrew the epithelium and from the latter there had differentiated distinct tubules with a lumen resembling sections of salivary glands. The necessity for a certain thickness of the mesenchyme in the outgrowth in order that differentiation may take place was noted independently by Rienhoff (1922). He found in cultures of the metanephros of the chick that the migrating renal cells, when in thin, flat outgrowths, formed a thin, flat sheet of cells with no further organization or differentiation. In cultures where a considerable thickness of the mesenchymal outgrowth had preceded the outgrowth of renal epithelium, the collecting tubules grew into this marginal zone as tubules, each one carrying undifferentiated nephrogenic tissue, which continued to differentiate into an S-shaped convoluted tubule and glomerulus, which finally joined the collecting tubule. Rienhoff also followed the differentiation in the thick migratory zone of blood sinuses, blood islands, and capillaries from primitive undifferentiated endothelial tissues. Drew (1923*a*) also found that when connective tissue was added to pure cultures of alveolar, mammary, carcinoma, and kidney epithelium, differentiation took place, otherwise the epithelial cells remained in sheets without further differentiation, thus confirming his previous observations (Drew, 1922) that the degree of differentiation shown by cultures of normal and malignant tissues is partly conditioned by the accompanying growth of stroma. The influence of connective tissue in cultures with epithelial cells in preventing the dedifferentiation of the latter was recognized long ago by Champy (1914*d*). Maximow (1923*a*) found in cultures of adult lymph nodes in plasma plus bone-marrow extract that some of the lymphocytes in the zone near the explant were transformed into typical plasma cells. He also claims that the myelocytes differentiated from both the large and small lymphocytes. M. R. Lewis (1917) observed the differentiation of connective-tissue fibrils in the ectoplasm of the connective-tissue cells found in cultures of the subcutaneous tissue from nine- to twelve-day chick embryos. Shipley (1916) found that erythrocytes develop from hemoglobin-free cells, and that when the region of the anlage of the heart of chick embryos after about twenty-four hours' incubation was explanted, before blood islands had appeared in the area opaca, heart muscle developed and exhibited contraction.

4. *Differentiation in explant:*

Harrison (1911) found that pieces of undifferentiated embryonic frog tissue will live for weeks in lymph, and undergo at least the initial stages of normal histological differentiation; cells from the axial mesoderm give rise to striated-muscle fibers; epidermal cells form a cuticular border; typical chromatophores and a mesenchymal-like tissue are formed from pieces containing portions of the neural tube and axial mesoderm. The primitive nerve cells in the walls of the neural tube and in the primordia of the cranial ganglia likewise underwent differentiation, for they gave rise to long nerve fibers. The most carefully followed instance of differentiation in the explant is that of the metanephros of the chick (Rienhoff, 1922). The living explants are unusually transparent, and the migration of cells away from it during the first forty-eight hours or so thins it out and enables one to follow from minute to minute and hour to hour the growth of the collecting tubules and the differentiation of the primitive metanephrogenic tissue into the S-shaped convoluted tubule and glomerulus, and its final junction with the collecting tubule. The glomerulus, including its capillaries and blood elements, differentiates *in situ* from an undifferentiated cellular mass which completely fills the distal end of the differentiating convoluted tubule. There is no cup formation with subsequent ingrowth of capillaries. In some instances the differentiation, growth, and ultimate migration of white blood cells was observed to take place in certain endotheliumlined spaces of the metanephros. In the metanephros, then, there occurs not only a differentiation of cells but also of a part of the organ, a morphogenesis as well as a cytogenesis. In explants of adult lymph nodes Maximow (1923*a*) found all stages in the differentiation, from the embryonic-like syncytial reticulum to fibroblasts, reticular cells, polyblasts, large lymphocytes (rare) and giant cells.

5. *Dedifferentiation in migratory zone:*

Champy (1912, 1913*a*,*b*,*c*, 1914*a*,*b*,*d*, 1915, 1920) has been the chief exponent of the dedifferentiation of various types of cells to a common indifferent form comparable to the cells of the young blastoderm. He claims that migrating smooth muscle, kidney epithelium, thyroid, parotid, testicle, etc., all revert to a completely indifferent cell type. The rapidity of dedifferentiation he believes to be dependent on the rapidity of the cell division. It has not been possible to reconcile the observations of Champy with others, including our own. Smooth muscle, for example, retains its essential characteristics in our cultures for many days, in spite of the fact that the cells may undergo mitotic division. The kidney epithelium, as Rienhoff has shown, either retains its epithelial character or, if the environment is favorable, continues to differentiate. Ectodermal epithelium likewise retains its distinctive epithelial character even after two or three months of subcultures (Fischer, 1922*a*). The intestinal epithelium, even after many days of primary cultures, also retains its distinctive epithelial character. Liver cells from young chick embryos likewise retain their distinctive characteristics, and nerve cells, when they do migrate, are capable of giving origin to axis cylinders. In regard to striated muscle, however, there is apparently distinct indication of a dedifferentiation. The muscle fibers which migrate out from explants made up to a large extent of cross-striated fibers lack, for the most part, any indication of cross-striations, even in the fixed specimens (Lewis and Lewis, 1917*a*). These muscle buds arise mainly from the cut ends of cross-striated fibers and in a manner very similar to that in regeneration. The process of dedifferentiation does not proceed in cultures to such an extent as to render the muscle fibers and cells indistinguishable from other cells. They retain, up to the time of the death of the cultures, certain peculiar characteristics which enable one to distinguish them from the connective-tissue cells. A few of the migrating muscle fibers occasionally show cross-striations, but it is not known whether they migrated out as such or whether a redifferentiation occurred. Such partially dedifferentiated muscle fibers do not lose their power of contraction (M. R. Lewis, 1920*b*).

A very similar process of dedifferentiation occurs in cultures of cross-striated heart-muscle cells from chick embryos four to eleven days old. Most of the muscle cells which migrate out in our cultures form a reticulum or membrane-like structure. It is possible to observe rhythmical contractions in individual cells, or in groups of cells that show no cross-striations either in the living or fixed specimens. Only occasionally do migratory cells show cross-striations (W. H. Lewis, 1924).

Fischer (1922*b*) cultivated cartilage cells, taken from the eye of the chick embryo, in plasma plus embryonic extract. No growth took place for some days, even with repeated subcultures, and only after the hyaline substance liquefied did the small cartilage cells migrate. These free cells gained in size rapidly and after further cultivation became spindle-shaped and, by coming into contact with one another, formed thin membranes. Pure cultures of these cells, which multiplied in number, were carried on for over three months without any indication of the formation of the hyaline substance. Not only were the cartilage cells enlarged several times but great morphological and physiological changes appeared to have occurred in them. Is this to be looked upon as another case of dedifferentiation in which cartilage cells have lost the power to produce the hyaline substance, or is it an instance where the environment does not supply the necessary substances for the production of cartilage? Thus it is seen that while certain types of cells may show a certain amount of dedifferentiation, they do not return to a common indifferent type, as claimed by Champy.

XII. PHAGOCYTOSIS

1. *Particulate matter:*

Various types of embryonic cells, such as clasmatocytes, fibroblasts, endothelium, white blood cells, epidermal cells, alveolar cells of the lung, liver cells, kidney tubule cells, endodermal cells, pigment cells of the retina, and flattened-out smooth-muscle cells, readily ingest particulate matter in the cultures.

The first phase in the ingestion of melanin pigment granules, for example, is, according to Smith (1921), the accidental contact of the granule with the cell and the adhesion of the two. The cells do not send out pseudopodia to inclose the granule but the latter appears to sink into the substance of the cell. When free in the surrounding medium such granules move about quite actively; they become quiet when attached to the surface of the cell, and after entering exhibit the same type of movement as that displayed by the neutral-red granules.

Of special interest are Smith's observations on the relation of the ingested granules to the preformed vacuoles of the cells. The usual conception as expressed by Shipley (1919) and others is that ingested particulate matter either finds its way into preformed vacuoles or vacuoles form about such particles as they enter the cell. Smith's observations, as well as our own, show quite clearly that ingested granules enter directly into the homogeneous cytoplasm and not into preformed vacuoles and that vacuoles do not form about such particles coincident with their entrance into the cell. Within three to twenty-four hours after the ingestion of melanin pigment granules by the fibroblasts, vacuoles began to show about them, first as a slight halo that gradually enlarged into a typical vacuole with the same affinity for neutral red shown by the degeneration vacuoles. Within these vacuoles the granules were slowly digested. Sometimes a single vacuole formed about a clump of three or four granules. The clasmatocytes ingested the melanin pigment granules more rapidly and in greater numbers, and vacuoles appeared to form about them more quickly than with the fibroblasts. In these cells, also, the granules do not appear to enter preformed vacuoles. The white blood cells did not ingest the pigment granules in such numbers or as rapidly as did the clasmatocytes.

2. *Bacteria:*

Smyth (1915*a*) found that embryonic chick tissues (mesenchyme, epithelium, and lymphocytes) growing in chicken plasma phagocytized freely human tubercle bacilli. Some of the cells digested the bacilli while others were killed by them, although human tubercle bacilli are supposed not to be pathogenic for chicken tissue. Somewhat similar results were obtained by Smith, Willis, and Lewis (1922) with avian tubercle bacilli added to cultures of embryonic chick tissue in Locke-bouillon-solution. The bacilli accumulated in very much greater numbers and more rapidly in the leukocytes and clasmatocytes than in the mesenchyme cells, but were destroyed more rapidly by the latter. Moreover, the leukocytes and clasmatocytes were destroyed long before the mesenchyme cells. The tubercle bacilli were also ingested by endothelial cells, ectodermal cells, liver cells, and kidney tubule cells, but not by striated muscle, nerve fibers, red blood cells, or ciliated epithelium. These investigators claim that phagocytosis is probably a physical phenomenon. The only bacilli that were taken in by the cells were those that accidentally came into direct contact with the cytoplasm throughout their entire length. The cells did not send out processes around the bacilli nor exhibit any behavior that could be interpreted as purposeful; all that could be seen was that a bacillus remained stuck to the cell for varying periods of time after which it suddenly was moved back and forth within the cytoplasm in the same manner as that exhibited by ingested pigment granules or by neutral-red bodies. It was found that the bacilli were taken directly into the cytoplasm and never into pre-existing vacuoles, although the latter might be quite numerous, nor was a vacuole formed around a bacillus at the time of its entrance into the cell. When first taken in, the bacilli were easily distinguishable from other cytoplasmic inclusions by their shape, index of refraction, and behavior. As they were moved back and forth within the cell, they gradually became paler, lost their even contour, and appeared as somewhat granular rods. At the same time a slight halo (a beginning vacuole) developed around each one. Within one to three hours shiny granules appeared in the bacillus, while the main body of the latter became paler and more difficult to recognize. After two or three hours the bright granules became small and pale and disappeared from sight. The contents of these vacuoles which develop around the bacilli and the bacilli themselves during certain stages of their digestion can be stained with neutral red. Sometimes only a portion of a bacillus takes up the stain.

The behavior of cells toward bacillus radicicola was studied (M. R. Lewis, 1923), because this organism is supposed to be able to live within the plant cells. The results were practically the same as with the avian tubercle bacillus. The cells ingested varying numbers of these organisms, depending upon the evenness of the suspensions, and rapidly digested them. Vacuoles were seldom formed around this organism, and it faded away in the homogeneous cytoplasm without causing a perceptible change in the latter.

XIII. TOLERANCE AND ACQUIRED TOLERANCE

In cultures it is possible to test directly the ability of various types of cells to withstand poisons and toxins and to acquire an increasing tolerance for them without the complications of intercellular fluids and blood, since the poisons to be tested are in direct contact with the cells. Embryonic mesenchyme cells, cultivated in fluid media with weak solutions of copper sulphate or of sodium arsenite, develop in the course of two days an acquired tolerance for strong doses of these two poisons (Wilson, 1922). Similar cultures with ethyl alcohol show that mesenchyme cells which have been growing for two or three days in a 3 per cent solution of the alcohol withstand stronger alcoholic solutions (25 to 40 per cent) for a longer period before death ensues than do control cells cultivated in an alcohol-free medium.

Fibroblasts respond in a similar manner to the presence of foreign proteins. A small amount of protein, added to the culture medium, does not modify the rate of proliferation, while a large amount does, unless the fibroblasts have been previously cultivated in the presence of a small amount of the foreign protein, in which case the cells become immunized against its action (Fischer, 1922*d*).

There have been many investigations, not of a cytological nature, which describe the effects upon tissue growth of various pathological agents. These will be found in the bibliography.

XIV. STRUCTURE OF DEGENERATING CELLS

All types of cells in primary cultures, with the usual media, sooner or later show degeneration changes (Plate IX *a* and *b*). These changes appear much earlier and proceed faster in some media than in others. For example, in simple salt solutions they may be quite apparent within twenty-four to thirty hours, while in a Locke-bouillon-dextrose or a plasma medium several days may pass before degenerative changes are especially noticeable. These changes also appear earlier and progress faster in some types of cells, such as mesenchyme, than in others, such as heart muscle or ectoderm. In liver cultures the endothelium often degenerates before the liver cells, and in cultures of the amnion the smooth muscle usually dies before the ectoderm.

1. *Granules and vacuoles:*

Let us consider, for example, the changes which take place in an ordinary mesenchyme cell that has migrated out on the cover glass in a Locke-bouillon-dextrose medium. There is first of all a gradual accumulation of small granules in the cytoplasm, granules that are not present in the normal healthy cells. These have a marked affinity for neutral red, and can be followed more easily when thus stained. They often move back and forth, in a more or less jerky manner, between the region of the centriole and the periphery of the cell. Sometimes, when they strike a mitochondrium their progress is blocked for a short time or deflected. As the granules increase in number and size many of them accumulate near the nucleus about the centriole. As degeneration continues vacuoles make their appearance about the granules, a vacuole inclosing one or several granules. They are very small at first but gradually increase in size and number and tend to accumulate about the centriole and enlarging centrosphere. These vacuoles move very much as do the granules until they become fairly large and the cells become crowded with them. Vacuoles or portions of the vacuoles often become stretched out into long threads. Although the contents of these vacuoles and the granules have a marked affinity for several of the vital dyes, such as neutral red, brilliant cresyl blue, and methylene blue, it seems rather a misnomer to call them a segregation apparatus, as Evans and Scott (1921) have done. Ingested particles and bacteria do not enter preformed vacuoles, and the affinity of the vacuoles for the vital dyes is an accidental affair, for they develop quite independently of them. They appear to us to arise from autolysis (self-digestion of the protoplasm) or from an accumulation of waste products, perhaps a combination of the two processes. Their affinity for the vital dyes indicates they are something more than imbibition of water. Many of the vacuoles develop about the granules, but we are uncertain whether vacuoles develop independently of the latter. In cells that become quite filled with vacuoles the cytoplasm is reduced to a slender framework, and since there is no marked increase in the size of the cells the vacuolar formation would seem to take place at the expense of the cytoplasm rather than by an imbibition of fluid from the outside. The rapid vacuolization induced by certain bacilli and toxins seems likewise to take place at the expense of the cytoplasm (Plate IX *c*).

2. *Mitochondria:*

As the granules and vacuoles increase in number, the long, threadlike mitochondria tend gradually to take on a definite radial arrangement about the centriole and slowly enlarging centrosphere. This arrangement is often quite marked and is partly due, perhaps, to the position of the mitochondria in the cytoplasmic framework which lies between the vacuoles. As degeneration progresses the mitochondria usually break up into rods and granules, and in extreme cases they may swell up into small vesicles. Under certain conditions they may remain as threads or rods, even though the cells become quite highly vacuolized. As a general rule, however, the threads are reduced to rods and granules, some quite minute, and the total amount of mitochondrial substance seems to decrease at the same time.

3. *Centrosphere:*

Parallel with the accumulation of granules and vacuoles, important changes take place in the region of the centriole. As already noted, the granules and vacuoles tend to become heaped up about the centriole and not about the nucleus. During this process a clear area develops about the centriole, pushing away the granules, vacuoles, and mitochondria from it. As degeneration goes on this clear area (centrosphere or central body) may gradually enlarge and attain a diameter equal to or even greater than that of the nucleus (W. H. Lewis, 1920*b*). The giant centrospheres usually present a clear medullary zone immediately about the centriole and a much wider cortical zone. The medullary zone, when it first appears, is surrounded by a zone of fine granules; as the cortical zone develops, most of the granules seem to be pushed to its periphery, but a few usually remain for a while between the two zones. These may ultimately disappear and with them all distinguishing boundary between the two zones. Sometimes the centrosphere contains minute, dustlike neutral-red granules, and in extreme cases may become vacuolated. In this giant-centrosphere type the periphery of the centrosphere, usually rather sharply marked off from the cytoplasm, is surrounded by degeneration granules and vacuoles and mitochondria. The latter lose their radial position, and become more or less concentrically arranged about it. In this type vacuolization of the cytoplasm is not usually extreme, but in cells where it is so (vacuolar type) the border of the centrosphere seems to merge into the surrounding cytoplasmic network lying between the vacuoles, and the mitochondria retain their radial arrangement. The two types are not sharply separated, for all transitions between them are found.

The accumulation of granules and vacuoles, as well as the changes in the mitochondria, occur in all types of embryonic cells observed in tissue cultures, such as mesothelium, endothelium, smooth muscle, striated muscle, heart muscle, ectoderm, endoderm, and liver cells. The giant centrosphere, however, has been observed only in mesenchyme, mesothelium, and endothelium.

4. *Nucleus:*

The nuclear changes taking place in these degenerating cells have not been studied as carefully as the cytoplasmic ones. All stages of direct division of the nucleus into two or several unequal parts are met with, especially in endothelial cells. Such stages are more frequently seen in fixed specimens, and it is very rare indeed actually to observe complete direct nuclear division. The binucleate cells, resulting from mitotic division of the nucleus without cleavage of the cytoplasm, frequently occur in various cell types. Since the binucleate condition in cells of the older cultures probably arises by direct division of the nucleus as well as by mitotic division, we are inclined to consider it as an indication of lowered vitality. Some degenerating binucleate cells have one centrosphere and some have two. It would be interesting if the two nuclei in cells with one centrosphere were the result of direct division, and those in cells with two centrospheres came from mitotic division. The multipolar spindles occasionally observed probably indicate degenerative conditions. In extreme degeneration the nuclear sap may be squeezed out into clear vesicles, leaving behind a small, irregular, deeply stainable pycnotic nucleus. In other cases one end of the nucleus may liquefy and the nuclear wall apparently break down, allowing the nucleoli to escape (W. H. Lewis, 1922*b*). In some cultures the various types of degenerating cells remain more or less spread out and adhere closely to the cover glass; in these the changes can be followed much more easily than where the cells retract their processes and round up. This rounding-up of cells during degeneration is quite common. Such cells often send out bleblike processes with fluid contents containing fine granules in Brownian movement. These rounded cells often fall to the bottom of the drop.

XV. CELL DEATH

The degenerative changes are followed sooner or later by the death of the cell unless the medium is renewed. There are certain visible irreversible alterations which indicate cell death. These may take place quite suddenly, as in fixation, or be prolonged, as when the cells die in cultures. Under the latter conditions the irreversible changes occur in fairly definite sequence and slowly enough to be followed. With weak fixatives and poisons the sequence can also be followed. The first noticeable change seems to be in the nucleus; the clear homogeneous nucleoplasm becomes cloudy and, as gelation proceeds, definitely granular. Before these changes begin it is impossible to see a definite nuclear membrane; after they are underway, however, one appears, and gradually becomes thicker. If a vital dye is present in the medium the nucleolus, which has been colorless, begins to take up the dye. If the mitochondria have previously been stained with Janus green they lose their color and disappear. This usually occurs after the nucleolus has begun to take up color. If the degeneration granules and vacuoles have previously been stained with neutral red or brilliant cresyl blue they likewise lose their color, one after another, and disappear. As a rule, the cytoplasmic changes follow the first nuclear ones, accompanying those of the mitochondria and granules. They usually begin in the long processes and at the periphery and proceed toward the center of the cell. The first is the appearance of minute granules in the cytoplasm. These do not take up the neutral red as do the degeneration granules. Finally, the cytoplasm becomes completely filled with them, all cell motion stops, and the cell has passed into an irreversible condition. Both nucleus and cytoplasm now stain diffusely with the various vital dyes, if in strong enough concentration; it is the minute granules due to the gelation process that take the stain. All these changes can be followed somewhat better with the dark field (W. H. Lewis, 1923*c*). Somewhat different sequences of changes may be produced by various other methods, but so far as we know they have not been so carefully followed.

The factors that bring about cell degeneration and death are somewhat difficult to analyze. We know that when the cultures are washed and the medium renewed the cells remain in a fairly healthy condition for a long period of time, but they ultimately die out unless embryonic extract is added to the medium, when the connective-tissue cells will live and multiply for an indefinite length of time in subcultures. Such factors as lack of proper food, salts, and oxygen, the accumulation of waste products, and changes in the H-ion concentration probably play the most important rôles.

PLATE I

a. Subcutaneous mesenchyme, two-day culture, eight-day chick embryo; Locke-bouillon-dextrose; Janus green, iodine. ×480.

b. Subcutaneous mesenchyme, three-day culture, eight-day chick embryo; Locke-bouillon-dextrose; Janus green, iodine. ×480.

c. Mesothelium, three-day culture from stomach, six-day chick embryo; 0.9 per cent NaCl; Janus green, iodine. ×480.

PLATE I

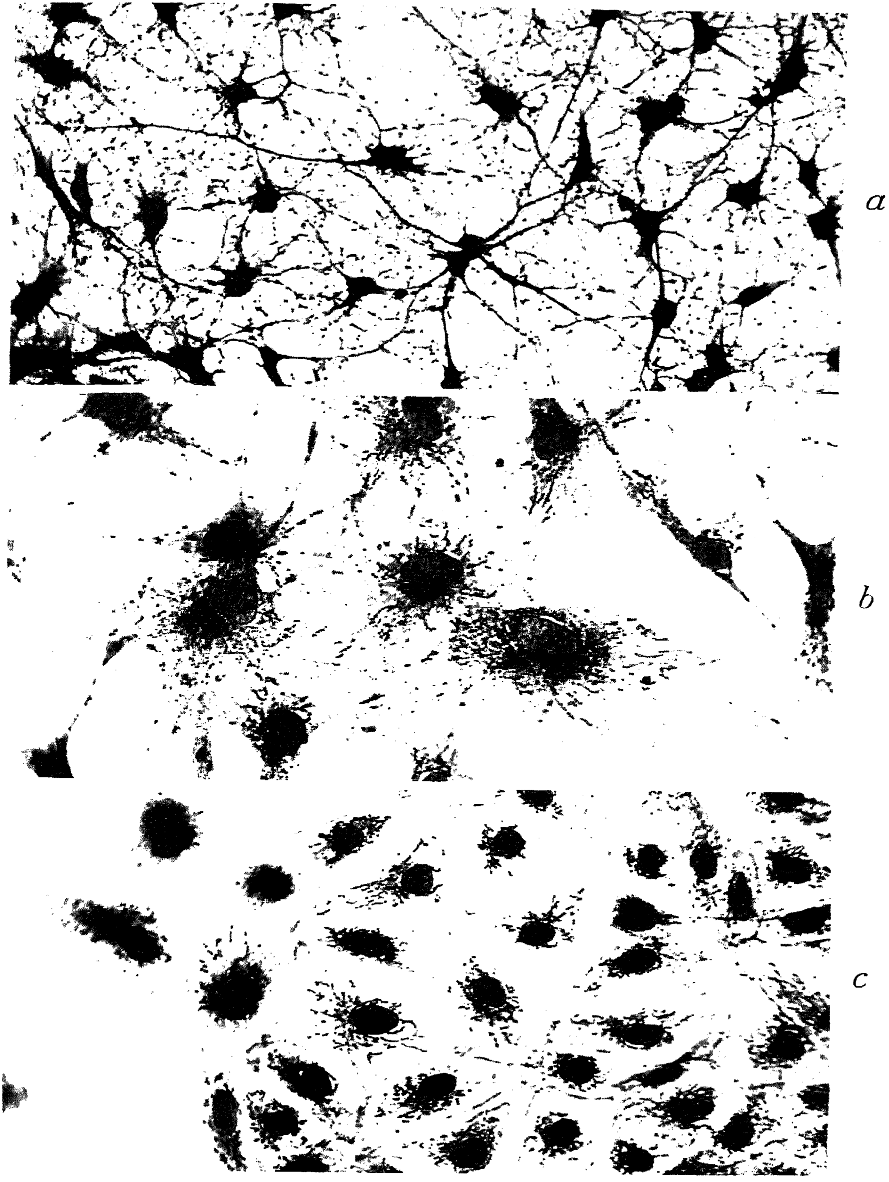


PLATE II

a. Liver cells and endothelium, seven-day culture from liver, ten-day chick embryo; Locke-bouillon-dextrose; neutral red, iodine. ×480.

b. Endoderm and mesenchyme, four-day culture of intestine, seven-day chick embryo; Locke-bouillon-dextrose; neutral red, iodine. ×146.

c. Isolated liver cell with mitochondria, six-day culture, eight-day chick embryo; Locke-bouillon-dextrose; Janus green, neutral red, iodine. ×1,450.

d. Endodermal cells with mitochondria mostly granules and rods, five-day culture from intestine, eight-day chick embryo; Locke-bouillon-dextrose; Janus green, iodine. ×1,450.

PLATE II

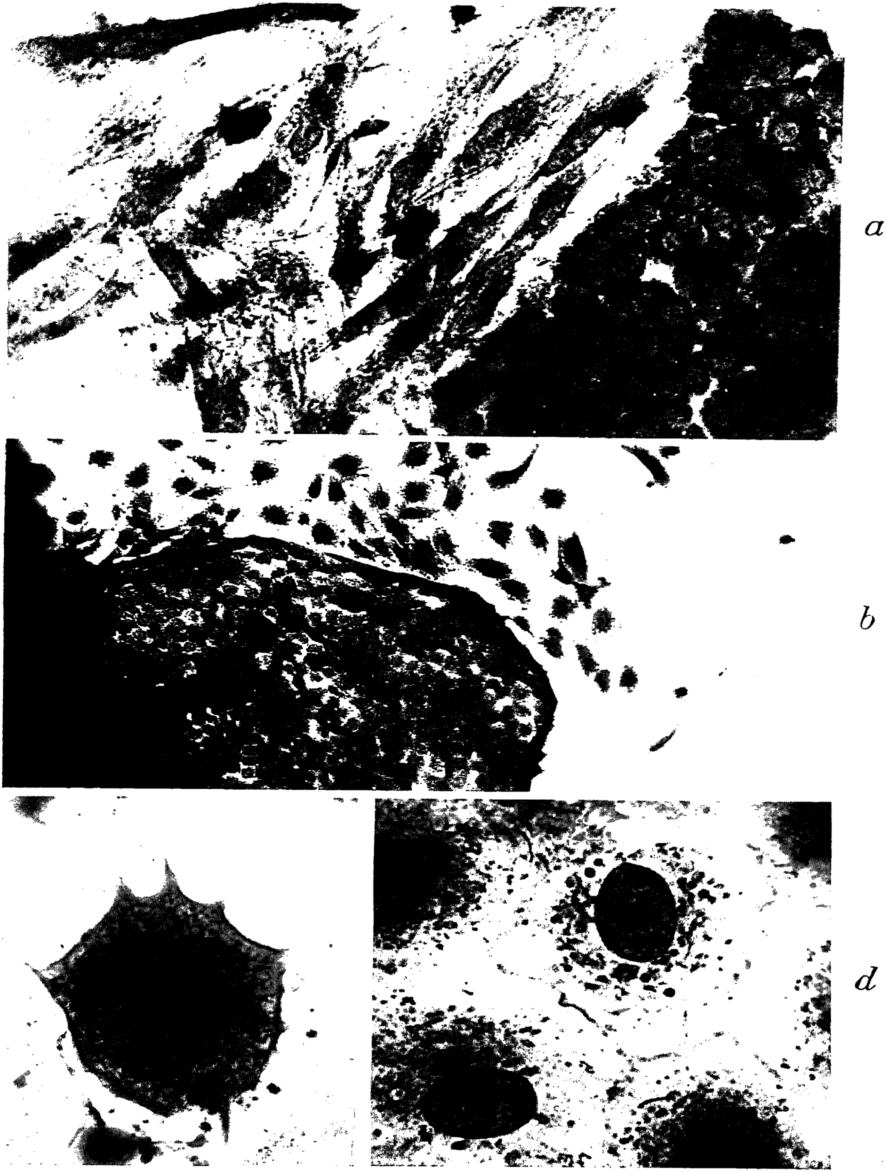


PLATE III

a. Ectoderm and smooth muscle, five-day culture from amnion, eight-day chick embryo; Locke-bouillon-dextrose; Janus green, neutral red, iodine. ×146.

b. Flat ectodermal cell with granular mitochondria, eight-day culture from amnion, seven-day chick embryo; Locke-bouillon-dextrose; Janus green, iodine. ×1,450.

c. Ectodermal cell with mitochondrial threads, six-day culture from amnion, seven-day chick embryo; Locke-bouillon-dextrose; Janus green, neutral red, iodine. ×1,450.

d. Ectodermal cell at edge of membrane with mitochondrial network, seven-day culture amnion, six-day chick embryo; Janus green, neutral red, iodine. ×1,450.

PLATE III

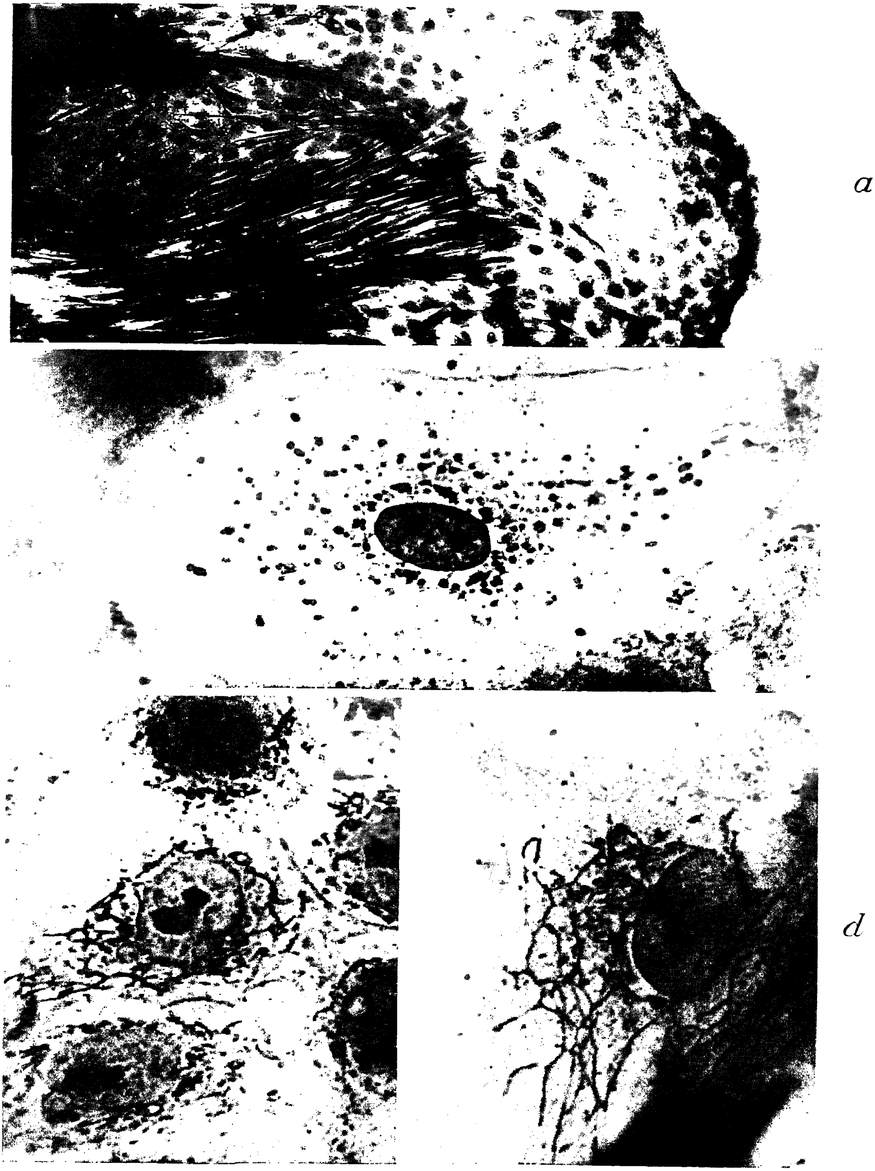


PLATE IV

a. Endothelial cell with striae and mitochondria, three-day culture from liver, nine-day chick embryo; Locke-bouillon-dextrose; Janus green, neutral red, iodine. ×1,030.

b. Smooth muscle with marked striae, one-day culture from amnion, ten-day chick embryo; Locke-bouillon-dextrose; Janus green, neutral red, iodine. ×480.

c. Smooth-muscle cell from preceding culture. Marked tension striae in three directions. ×1,450.

PLATE IV

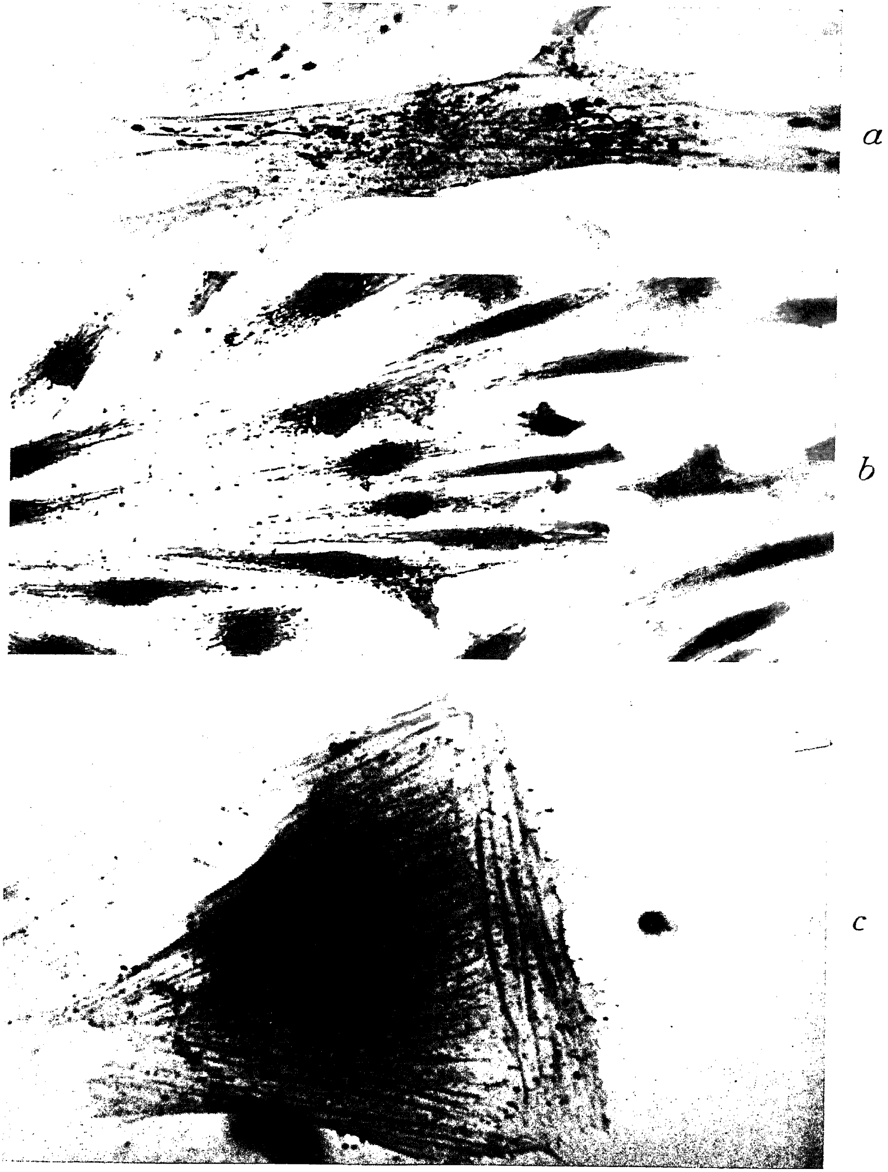


PLATE V

a. Smooth-muscle cell with tension striae, mitochondrial short threads, four-day culture from amnion, six-day chick embryo; Locke-bouillon-dextrose; Janus green, neutral red, iodine. ×1,450.

b. Mesothelial cells with tension striae, granular mitochondria and large centrosphere, two-day culture from heart, six-day chick embryo; 0.9 per cent NaCl; Janus green, iodine. ×1,450.

c. Growing end of sympathetic nerve fiber, six-day culture from mesonephros, ten-day chick embryo; Locke-bouillon-dextrose; Janus green, neutral red, iodine. ×1,450.

d. Tumor cell, two-day culture of mouse sarcoma No. 180; Drew saline plus embryonic extract; neutral red, iodine. ×1,450.

PLATE V

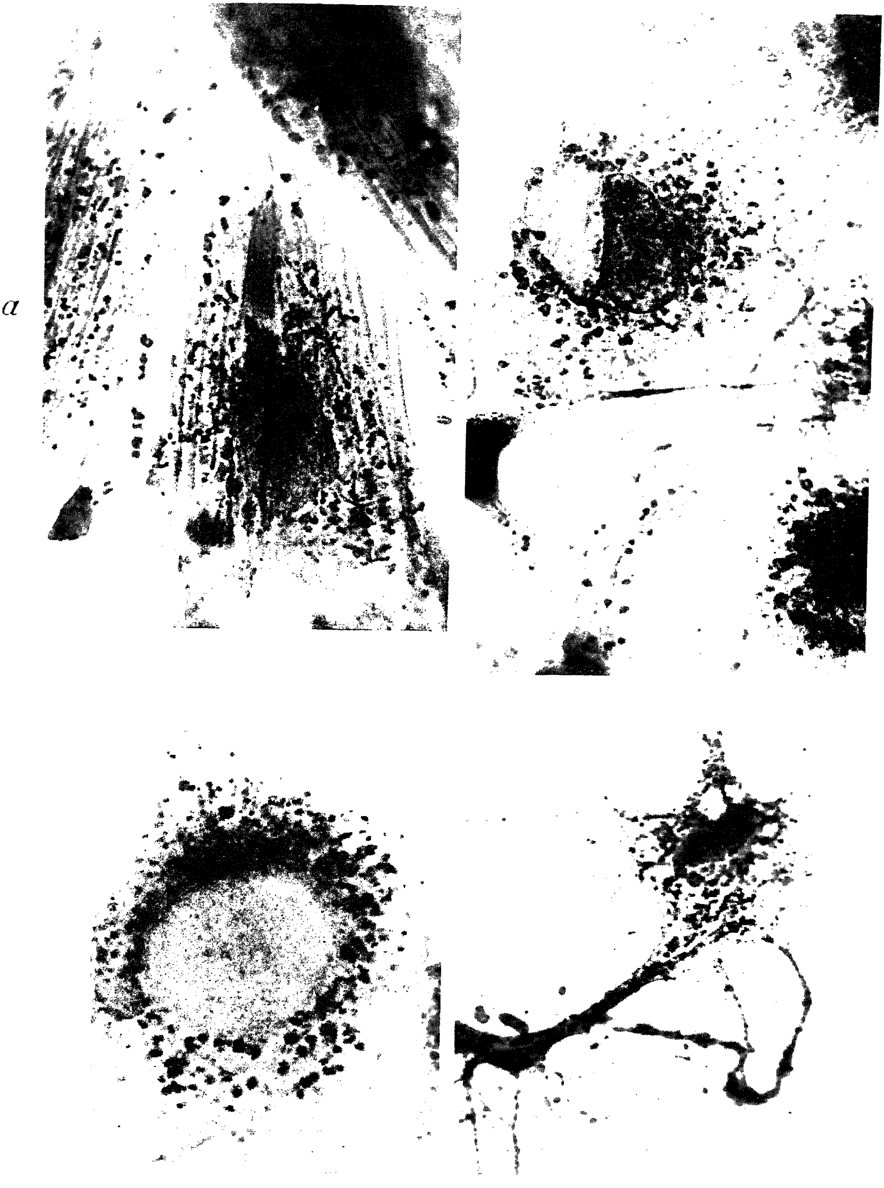


PLATE VI

a. Heart muscle and loose mesenchyme, five-day culture from heart, four-day chick embryo; Locke-bouillon-dextrose; iodine, ×146.

b. Heart muscle cell, seven-day culture, four-day chick embryo; Locke-bouillon-dextrose; Janus green, iodine. ×1,450.

c. Two heart-muscle cells from same culture; each cell was pulsating at a different rate. ×1,450.

PLATE VI

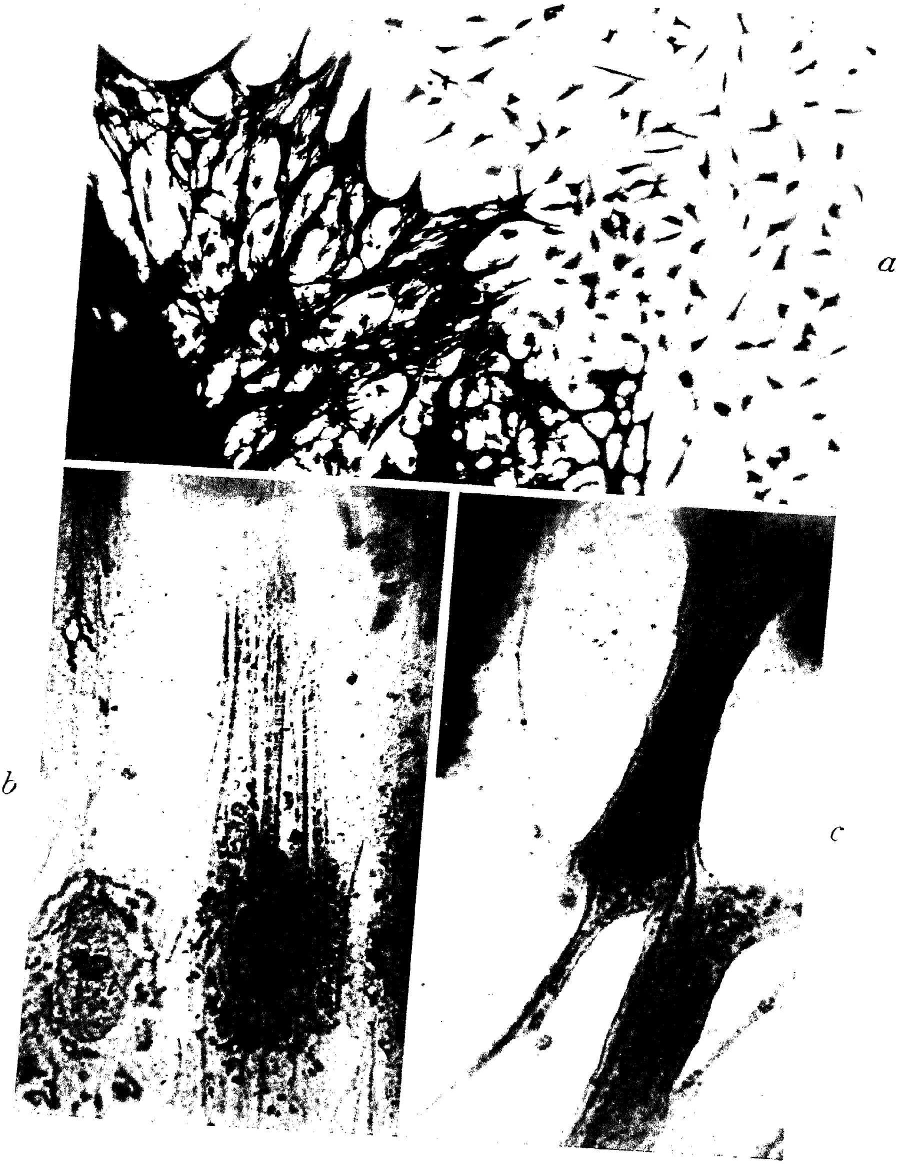


PLATE VII

a. Skeletal muscle, two-day culture, from leg of eight-day chick embryo; long multinucleated muscle strands with expanded tips; Locke-bouillon-dextrose; hematoxylin and eosin. × 140.

b. Skeletal muscle, two-day culture from wing of eight-day chick embryo; Locke-bouillon-dextrose; hematoxylin and eosin. × 100.

c. Cross-striated fibers, five-day culture, ten-day chick embryo; Locke-bouillon-dextrose; hematoxylin and eosin. × 1,000.

PLATE VII

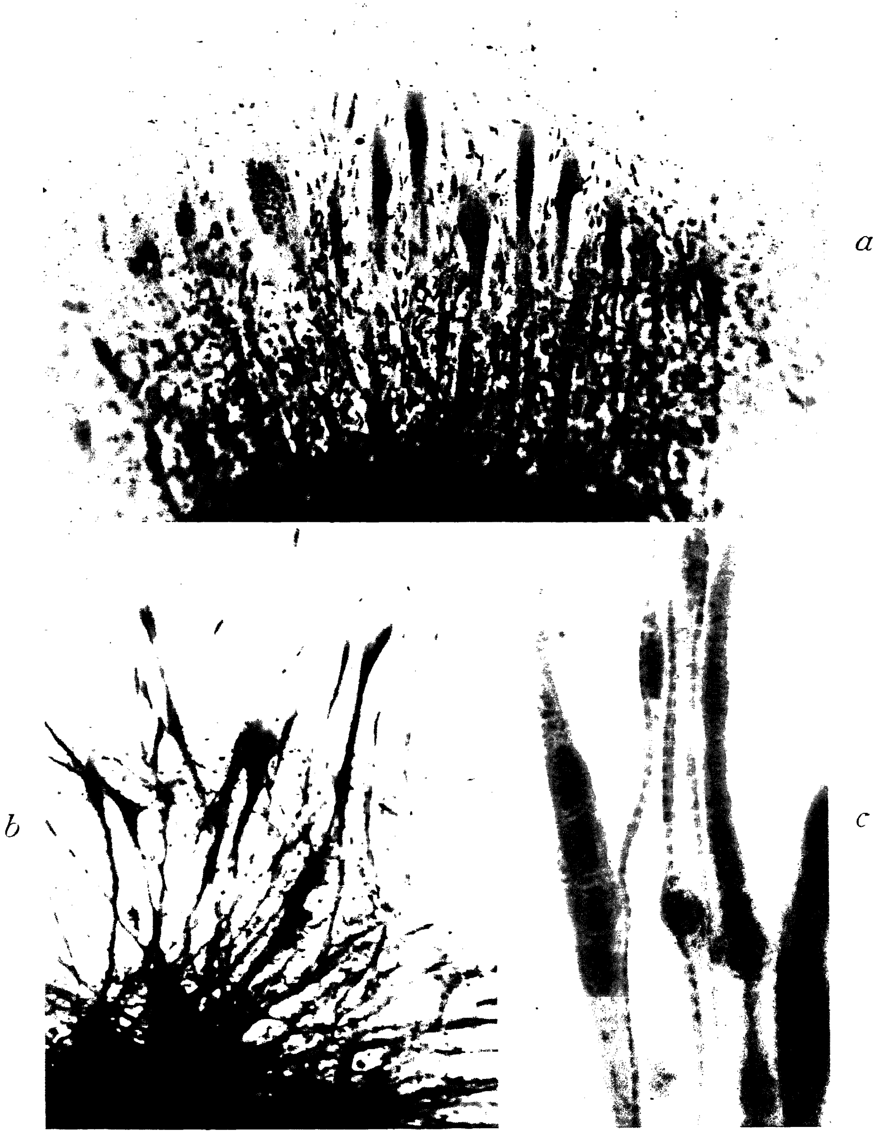


PLATE VIII

a. Giant cells, endothelioid cells and lymphocytes, four-day culture from tuberculous human lymph node; plasma; hematoxylin and eosin. ×146.

b. Migrating lymphocytes and endothelioid cells with ingested lymphocytes, two-day culture from tuberculous human lymph node; plasma; hematoxylin and eosin. ×480.

c. Dead lymphocytes, endothelioid and connective-tissue cells with ingested lymphocytes, four-day culture from tuberculous human lymph node; plasma; hematoxylin and eosin. ×480.

PLATE VIII

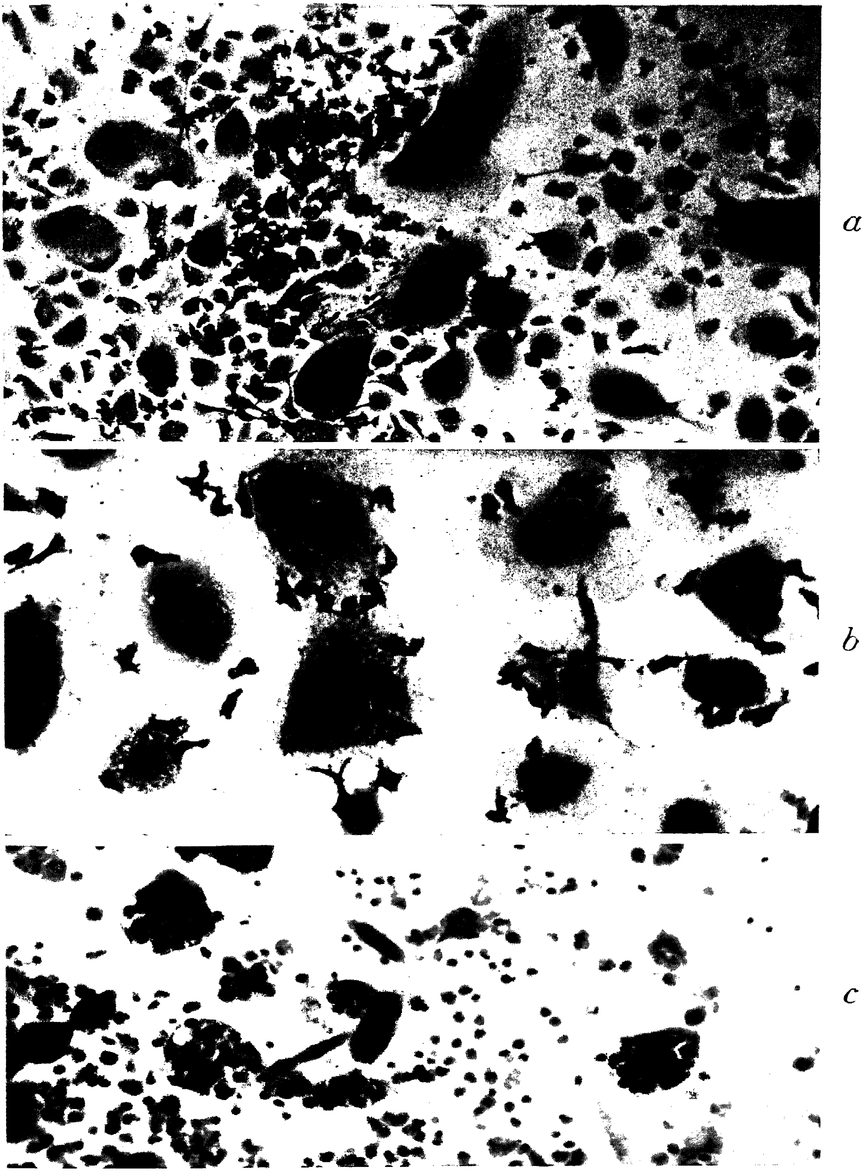


PLATE IX

a. Binucleated mesenchyme cell highly vacuolated, mitochondrial rods and granules; nine-day culture from stomach, six-day chick embryo; 0.9 per cent NaCl; Janus green, iodine. ×1,450.

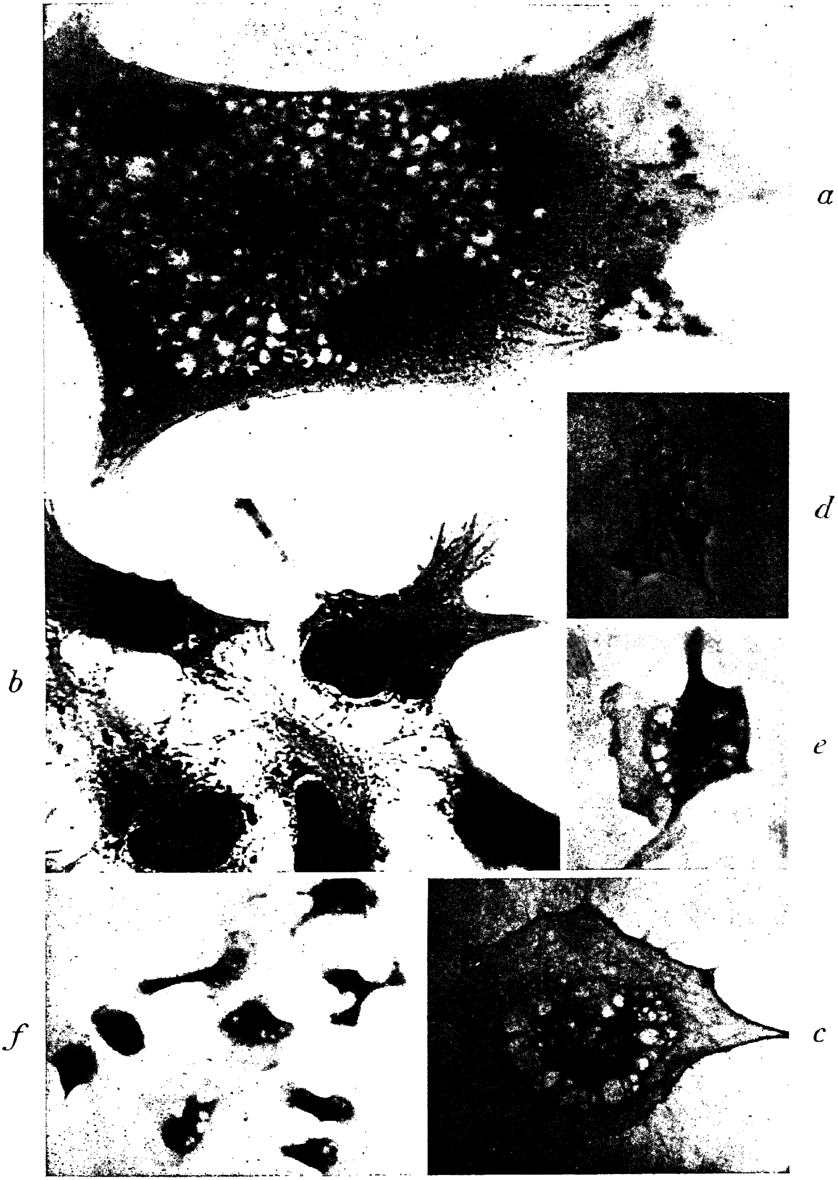
b. Mesenchyme cell with lobulated nucleus, large central area surrounded by mitochondrial rods and granules; three-day culture from stomach, six-day chick embryo; 0.9 per cent NaCl; Janus green, iodine. ×1,450.

c. Vacuoles produced in two and one-half hours by typhoid bacilli in mesenchyme cell, two-day culture from intestine, eight-day chick embryo; Locke-bouillon-dextrose; hematoxylin and eosin. ×550.

d. Clasmatocytes, two-day culture of subcutaneous tissue, eleven-day chick embryo; Locke-bouillon-dextrose; hematoxylin and eosin. ×1,450.

e–f. Clasmatocytes, two-day culture of subcutaneous tissue, twelve-day chick embryo; Locke-bouillon-dextrose; hematoxylin and eosin. *e*, ×1,450; *f*, ×480.

PLATE IX



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SECTION VIII

FERTILIZATION

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FERTILIZATION

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Fertilization is the union of two sexually differentiated gametes to form a single cell, the zygote. The processes of fertilization may, therefore, logically be held to include the interactions of the ripe gametes prior to their union, and the phenomena involved in their union up to the time when both morphological and physiological individuality of the zygote is attained. To limit the use of the term fertilization to the first part of the interaction of the gametes, as some would do, goes contrary to long usage; to extend it, as others have suggested, through the life-history up to the period of synapsis of the maternal and paternal chromosomes destroys the unity of the conception. The period of fertilization as defined above includes a wide range of cytological phenomena, both morphological and physiological; some of these are unique in the same sense that the entire process is a unique one in the life-history, and others belong to more general cytological categories. It will be logical, therefore, to concentrate our attention as far as possible on the more specific characters of the period under discussion.

The essential feature of fertilization, viz., the fusion of two cells into one morphological and physiological unit is found in no other biological phenomenon. The activation of processes that accompanies the union, on the other hand, has its analogies in various processes of stimulation and response in diverse cells of the organism. So far as the egg is concerned, moreover, activation may be induced by various artificial means, or it may occur normally without fertilization, in parthenogenesis. The phenomena of parthenogenesis, however interesting in themselves, are not fertilization and they will, therefore, receive no systematic treatment. But as the mechanism of activation of the egg is the same, whether stimulated by fertilization or by artificial parthenogenesis, the results of the latter method of investigation may be utilized for purposes of interpretation of this part of the subject of fertilization.

I. MORPHOLOGY OF FERTILIZATION

1. *Relation of fertilization to the state of maturation of the ovum:*

In all species which are not naturally parthenogenetic, the ovum after attainment of its full growth reaches a stage of quiescence or of inhibition in which it remains until it dies, unless it be fertilized or otherwise effectively stimulated by parthenogenetic agents. This stage is usually constant for any given species but varies in different animal groups. The following principal conditions all pertaining to stages of the maturation divisions may be recognized: (1) The ovum is inhibited at the end of its period of growth. The large nucleus, known as the germinal vesicle, undergoes none of the preparatory stages of maturation unless the egg be fertilized; this is the case, for instance, in the annelid *Nereis* (see Fig. 1). (2) The ova of the annelid, *Chaetopterus*, of the nemertean, *Cerebratulus*, and of the lamellibranch, *Cumingia*, and presumably some other forms, pass through the prophases of the first maturation division, but the karyokinetic process is arrested in the mesophase of this division, and proceeds no farther until the egg is fertilized. (3) In the case of the ova of many vertebrates the first polar body is formed, and the prophase of the second maturation division begins, but the process then stops unless the egg be fertilized. (4) In the echinids and some other animals maturation is completed without fertilization.

In the first three cases the spermatozoön remains more or less quiescent within the egg during the completion of the maturation divisions, and the internal events of fertilization are renewed after the formation of the second polar globule. These

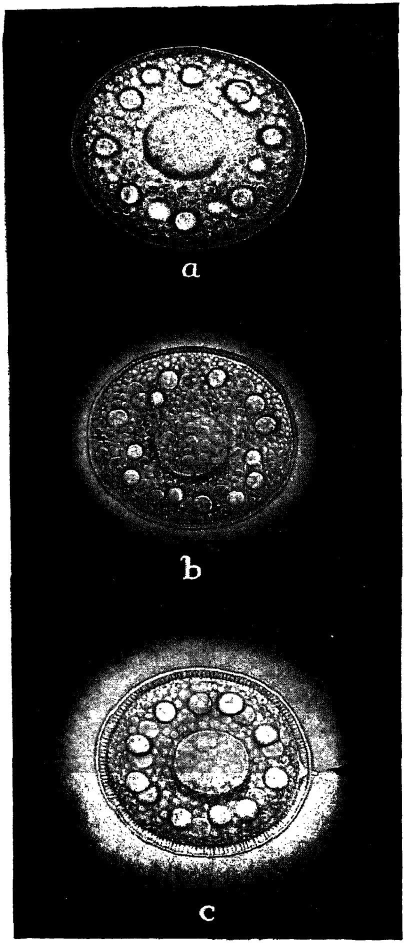

FIG. 1.—Drawings from photographs of *Nereis* eggs, in a suspension of India ink in sea water; (*a*) before insemination; (*b*) three minutes after insemination; (*c*) twelve minutes after insemination. (*a*) The uninseminated egg is bounded by a strong membrane; within this is a cortical layer without yolk granules, and of alveolar structure; (*b*) shortly after attachment of the spermatozoön (not shown in the figure) the egg extrudes a transparent jelly from the alveoli of the cortical layer; (*c*) the secretion of the jelly is completed; the walls of the emptied alveoli of the cortical layer now appear as radiating lines crossing the perivitelline space. The spermatozoön is seen (to right) with a cone of ink extending into the jelly where its tail lies; the protoplasm of the egg forms a fertilization cone which crosses the perivitelline space and touches the membrane beneath the spermatozoön.

variations of the time at which the egg reaches the period of inhibition or quiescence affect the morphological features of fertilization in certain important respects; they must also be borne in mind in the interpretation of experiments.

2. *Cortical changes of the egg and entrance of the spermatozoön:*

The first changes to be observed in the egg as a result of fertilization concern the membranes and the cortex, and are usually closely associated with the entrance of the spermatozoön; these may precede the entrance of the spermatozoön within the egg (e.g., *Nereis*, Fig. 1), but more usually such changes, considered in a purely morphological sense, follow entrance of the spermatozoön. They are quite variable in appearance in different eggs owing to structural and chemical differences of the eggs. If we conceive these changes broadly as response of a specific protoplasmic system to a stimulus, it is obvious that the nature of the response must be given in the system itself, and hence that different kinds of eggs may be expected to differ in the nature of their visible cortical changes.

A. THE CORTICAL CHANGES

We shall consider the eggs of echinids first because they are so favorable for a variety of physiological experiments, and are hence referred to frequently in this section. The unfertilized egg is surrounded by a layer of soft, transparent jelly about one-fourth the diameter of the egg in thickness; by observation alone it would be impossible to say whether the egg itself is bounded by a preformed membrane, but by the aid of micro-dissection and in other ways it is possible to demonstrate the existence of an exceedingly delicate vitelline membrane in immediate contact with the protoplasm proper (see Kite, 1912; Heilbrunn, 1915; Chambers, 1921).[1] The cortex of the egg is not clearly distinguishable morphologically from the endoplasm, but by various methods

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[1] J. Loeb (1909) accepts the existence of a differentiated surface lamella prior to fertilization (p. 125). This membrane becomes thicker and less permeable to spermatozoa after fertilization. In 1912 McClendon suggested that the fertilization membrane is a precipitation formed between two oppositely charged colloids, viz.: the egg jelly and a substance secreted from the egg. Elder (1912) also presented the view that the "membrane is merely a precipitation membrane formed as a result of a reaction which occurs between the outflowing fluid of the protoplasm, after entrance of the spermatozoön into the egg, or through the influence of some artificial agent and the inner surface of the zona as the latter passes into a state of solution" (p. 161). E. N. Harvey (1914) showed that normal membranes are formed by eggs entirely deprived of jelly, an observation confirmed since by several investigators (Lillie, 1914, 1921; Just, 1919; and others). Yet Gray (1922) returns to the McClendon-Elder hypothesis. The jelly may be removed from sea-urchin eggs either by shaking or dissolving it off in dilute acid. If the latter method is followed the greatest care has to be observed lest the acid attack the egg and destroy its surface. If sufficient care is observed in the acid treatment it is as easy to show by this method as by shaking that uninjured eggs deprived of the last trace of jelly form as perfect membranes on insemination as those with jelly.

(pp. 479, 492–93) it is possible to show that a cortical layer exists which differs in its physiological properties from the internal protoplasm.

Within one-half minute to about two minutes after insemination, varying according to temperature, and the physiological conditions of the gametes (discussed beyond), the delicate vitelline membrane becomes elevated from the surface of the egg and separated from it by a space of uniform width (*perivitelline space*) containing a clear fluid (Fig. 2). As the membrane first becomes clearly visible at the time of fertilization, it has been called the fertilization membrane. The elevation of this membrane is an unfailing indication of successful insemination. Within a few minutes after elevation of the membrane, a thin hyaline layer, apparently of protoplasm, forms on the surface of the egg. This has been variously known as "ectoplasm," "plasma film," or "gelatinous film" (Loeb).

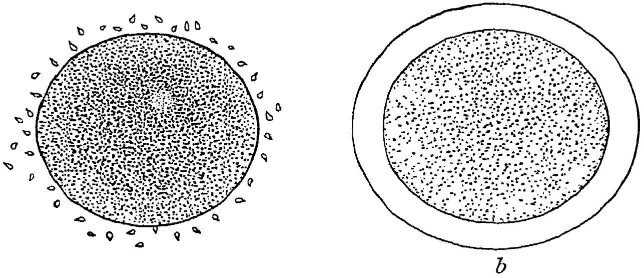

FIG. 2.—The formation of the fertilization membrane in the egg of the sea urchin *Strongylocentrotus purpuratus:* (*a*) unfertilized egg surrounded by spermatozoa; (*b*) the same egg about two minutes later after the entrance of the spermatozoön (from Loeb, *Artificial Parthenogenesis and Fertilization*, p. 17; by permission of the author).

More careful examination of the phenomena shows in the case of the egg of *Echinarachnius* (sand dollar) that the spermatozoön, which reaches the surface of the egg through the jelly, entirely ceases movement on attachment, and is engulfed by the egg protoplasm within thirty to fifty seconds after insemination (see Just, 1919*a*). After the spermatozoön has disappeared within the egg, the membrane begins to be elevated above the surface of the egg at the point of entrance, and is rapidly elevated in a wavelike progress from this point to the opposite point of the surface of the egg until it is entirely detached; it then becomes turgid and evenly separated from the surface of the egg at all points apparently through osmotic pressure of the perivitelline fluid. During the process of elevation droplets can be seen to pass from the cortex of the egg into the perivitelline space, rapidly dissolving in the fluid (Just, 1919*a*). After the membrane is elevated, a hyaline layer of protoplasm forms over the surface of the egg; this layer, frequently called ectoplasm, acts apparently as a semipermeable plasma membrane.

The first author to describe the fertilization membrane of sea urchins (Fol, 1876) also describes the formation of the membrane in a wave proceeding from the point of entrance of the spermatozoön; but in the sea urchins most studied, the process proceeds so rapidly that its wavelike origin has escaped the attention of most observers. In *Strongylocentrotus* also, Lillie has observed the progressive detachment of the membrane and has noted that in stale eggs the process may be incomplete so that the membrane remains permanently eccentric.

Just (1919*a*) observes that the point of entrance of the spermatozoön "becomes a 'point of injury' and is 'negative' for any other sperm arriving at this point, all other portions around the egg being positive." This "wave of negativity" then moves over the surface of the egg in advance of membrane elevation until the entire surface of the egg is sterile to other spermatozoa.

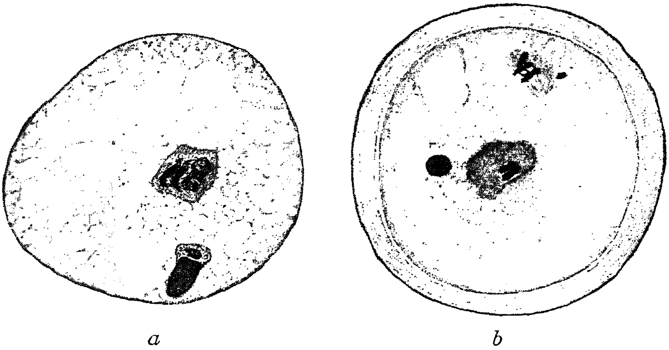

FIG. 3.—Entrance of the spermatozoön and formation of the fertilization membrane in *Ascaris megalocephala:* (*a*) The entire spermatozoön within the egg; central germinal vesicle with tetrads; the egg is membraneless. (*b*) The spermatozoön has reached the center of the egg and its cytoplasmic parts are disintegrating. First maturation spindle near the surface. A thick fertilization membrane has been formed, separated from the egg by a narrow perivitelline space.

In *Nereis* the unfertilized egg possesses a thick cortical layer of alveolar structure inclosing the yolk- and oil-containing endoplasm (Fig. 1*a*). When insemination takes place, a large number of spermatozoa become attached to the surface of the egg if the sperm is present in excess. In about two or three minutes all spermatozoa, with the exception of one, which is alone concerned in the subsequent processes of fertilization, begin to be carried away from the surface of the egg by an outflow of transparent jelly derived from the cortical alveoli which are gradually emptied, thus establishing a perivitelline space, crossed by the delicate protoplasmic walls of the empty alveoli. The outflow of jelly lasts for ten to twelve minutes. The cortical changes thus present a very different appearance from those of the sea-urchin egg.

The egg of *Ascaris megalocephala*, which is entirely devoid of a preformed membrane before fertilization, likewise secretes a gelatinous substance which rapidly hardens into a thick resistant membrane separated from the surface of the egg by a perivitelline space (Fig. 3). In the eggs of most animals a perivitelline space appears between the vitelline membrane and the surface of the egg as a result of insemination.

In all animals there is clear morphological evidence of cortical changes following immediately on insemination. It is obvious that as all environmental relations are mediated through the cortex, the changes described, indicating modifications of permeability, must be of great functional significance, a matter that is discussed in the physiological division of this section (pp. 492, 514, etc.).

B. THE ENTRANCE ("PENETRATION") OF THE SPERMATOZOÖN INTO THE EGG[1]

The entrance of the spermatozoön into the egg commonly takes place within a minute or less after contact with the egg (*Echinarachnius*, fourteen to forty-five seconds; Just, 1919*a*); but in the case of *Nereis* (described below) entrance is delayed until forty or fifty minutes after contact, during which time the cortical changes are completed and the maturation of the egg is begun. It was formerly supposed that the spermatozoön bored into the egg by virtue of its own motor activity; hence the term "penetration" of the spermatozoön. But it has been shown that this is not so, in the best-known cases at least, and that the egg actively co-operates and may actually be regarded as engulfing the spermatozoön. In the case of the eggs of many animals with thick resistant membrane one or more apertures, micropyles, exist in the membrane through which the spermatozoön enters (e.g., insects, teleosts). In small eggs, such as those of echinoderms, annelids, many mollusks, mammalia, etc., the spermatozoön may enter at any point; in larger teleolecithal eggs entry may be limited to the less deutoplasmic animal hemisphere (e.g., Amphibia).

In the simplest cases (morphologically), e.g., sea urchin and sand dollar, the spermatozoön disappears within the cortex of the egg without any previous noticeable reaction of the egg surface. In the case of the sand dollar a cone of clear protoplasm protrudes from the egg after the entrance of the spermatozoön at the point of entry. This may be called the "exudation cone" (after Fol) to distinguish it from the fertilization cone of other eggs described below. In the starfish, according to Chambers (1923), the blunt heads of the spermatozoa are unable to penetrate through the viscous jelly that surrounds the egg;

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[1] Exact observations on the entrance of the spermatozoön into the egg begin with Hermann Fol (1877, 1879), though this had been inferred previously by Prevost and Dumas (1824), Barry (1840), Newport (1854), Meissner (1855), Bütschli (1873), Auerbach (1874), Van Beneden (1875), O. Hertwig (1875). Fol's observations concerning the formation of the fertilization membrane and the entrance of the spermatozoön in the sea urchin and starfish were so accurate that practically no improvements were made on them until very recent times. Fol propounded the theory that prevention of polyspermy is due to the fertilization membrane.

they become entangled in the outer layers. The egg responds to the presence of the spermatozoa by forming one or several hyaline conical elevations on its surface ("fertilization cone," Fol). "From the summit of each cone a filamentous process grows out through the jelly until it touches and adheres to a motionless sperm head that happens to lie in its path" (Fig. 4). This filament then appears to retract, drawing the spermatozoön through the jelly to the cone. During this process the elevation of the fertilization membrane begins, and spreads from this point over the surface of the egg. In case there is more than one spermatozoön so captured the more advanced filament appears to inhibit the others, so that in healthy eggs only one spermatozoön reaches its cone. It then passes through the membrane into the cone (*d*) which is gradually retracted, and an exudation cone takes its place for a brief time. The time relations are given in the caption of the figure.

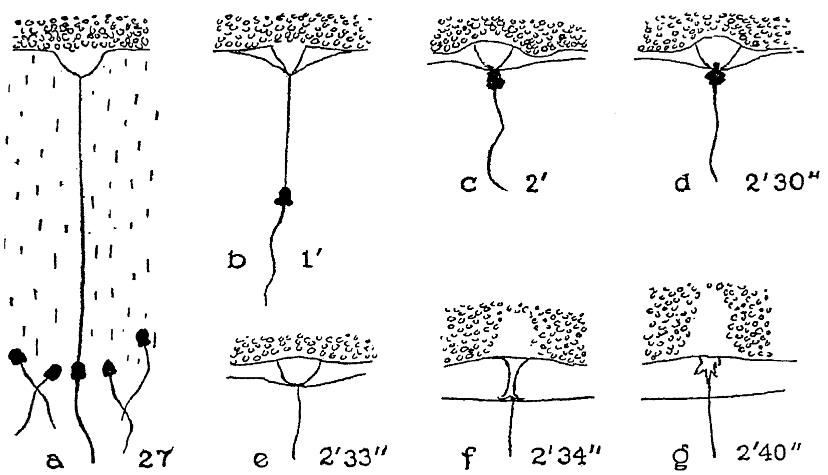

FIG. 4.—*a–g*, *Asterias forbesii*. Seven steps in the passage of a spermatozoön through the jelly and into the living egg. The whole process figured here lasts about 2½ minutes. (*a*) 27 seconds after insemination. From the fertilization cone extends a delicate filament to the periphery of the jelly where the tip of the filament has touched a sperm head. (*b*) 30 seconds later. The spermatozoön has been dragged halfway through the jelly. At the base of the cone the fertilization membrane is rising. (*c*) 1 minute later. The sperm head has reached the cone. (*d*) The tip of the sperm head is narrowing as it passes through the fertilization membrane into the cone. (*e*) The head is within the cone. (*f*, *g*) The sperm head is sinking into the egg and the fertilization cone is being replaced by what Fol termed the exudation cone (after Chambers).

This extraordinary process implies an action of the spermatozoa presumably by diffusion through the jelly upon the surface of the egg, unless the filaments are supposed to pre-exist in the jelly of the unfertilized egg, where, however, Chambers was unable to detect them. The protection against polyspermy in this case appears as a transmission effect from the most advanced cone and filament, though Chambers postulates a supplementary protective effect of the fertilization membrane also, in the sense of Fol.[1]

In the case of *Nereis* (Fig. 1*c*) a cone-shaped extension of hyaline protoplasm crosses the perivitelline space to touch the point on the membrane where the successful spermatozoön is attached. This arises about fifteen minutes after insemination, and lasts as a projection for only a few minutes; it persists, however, as a modified area of protoplasm which is concerned in the penetration of the spermatozoön as described below (Fig. 5).

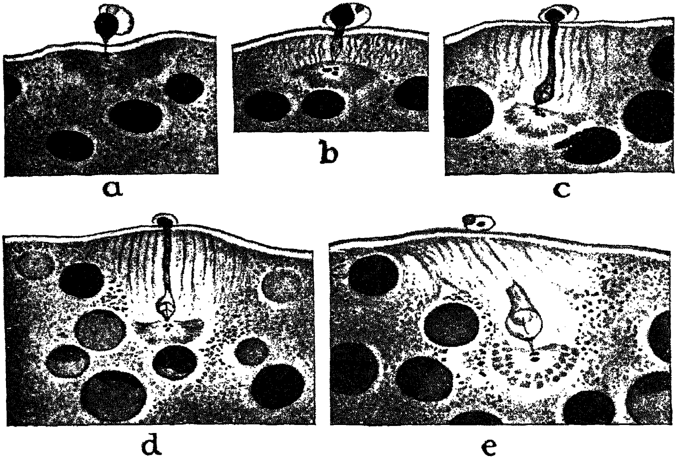

FIG. 5.—Penetration of the spermatozoön in the egg of *Nereis*, from sections: (*a*), 37 minutes after insemination; (*b*, *c*, *d*,) three stages from eggs killed 48½ minutes after insemination; note that the cone sinks into the egg and draws the spermatozoön after it; (*e*) 54 minutes after insemination; the head of the spermatozoön now entirely within the egg is contracting; note that the middle piece remains on the membrane; it does not enter the egg; the tail also remains outside.

Entrance of the spermatozoön is so rapid in most eggs that the details cannot be successfully observed. In *Nereis* the process is very slow; about fifteen minutes after insemination (Fig. 1*c*) the perforatorium of the spermatozoön has effected an opening through the vitelline membrane and its tip is imbedded in the fertilization cone. The cone then gradually flattens out and no more changes are seen for about thirty minutes. The head of the spermatozoön then passes rather abruptly through the membrane into the egg, but the tail and middle piece are left behind on the vitelline membrane. This happens about the time of the anaphase of the first maturation division.

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[1] Chambers' account of the entrance of the spermatozoön in the starfish egg is given literally above. It differs so radically from previous accounts and introduces so much difficulty in the way of interpretation that we are inclined to question whether the phenomena described are those of normal fertilization. Just (unpublished observations) finds that sections of eggs fixed five seconds after insemination show spermatozoa that have reached the surface of the egg through the jelly. Spermatozoa are well attached to the membrane fifteen seconds

Stained sections show the details of the final penetration of the sperm head very beautifully (Fig. 5). The complex made up of the head of the spermatozoön and the fertilization cone in which the perforatorium is imbedded act as a unit. The cone retreats into the interior of the protoplasm, and the viscous head of the spermatozoön is drawn through the aperture in the vitelline membrane as wire through a die. As fast as the nucleus, contained in the head, enters the protoplasm it begins to swell. When entrance is completed, the middle piece and tail of the spermatozoön are left behind on the vitelline membrane (Fig. 5*e*), where they remain and can be seen until after the first cleavage.

It is quite certain that in this act of penetration, the initiative, so to speak, is on the side of the ovum, which engulfs the fertilization cone, drawing the sperm head after it. These events can be understood by assuming that the spermatozoön causes a local diminution of surface tension of the egg in the first place, thus causing an outflow of protoplasm, the fertilization cone; and that then by subsequent coagulation in the cone, the surface tension of this region rises until it is overflowed by the surrounding protoplasm and sinks into the interior. The much more rapid penetration in other forms may possibly be understood on similar principles.

In other animals more of the spermatozoön enters the egg than in the case of *Nereis*. In sea urchins, according to the accounts of various authors, the head and middle piece enter, and the tail is left outside (cf. also Fig. 4*e*, *f*, *g*). But undoubtedly in most animals the entire spermatozoön enters as in nematodes, Crustacea, Mollusca, some insects, Amphibia, and mammals (Fig. 6). These differences are presumably of secondary importance, and they may be inessential, a matter that is discussed beyond. It is probable that the middle piece and tail are concerned primarily in accessory functions of fertilization, especially the motility that is necessary to bring the gametes together.

3. *The internal phenomena of fertilization:*

The morphological part of this subject consists in following the parts of the spermatozoön within the egg and determining as far as possible their relations

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after insemination; the membrane begins to form at the point of attachment of one spermatozoön at about forty-five seconds after insemination, and the cone arises in the space thus provided. The spermatozoön enters the cone about two minutes after insemination. These data are in serious conflict with part of Chambers' time-table (Fig. 4). We believe that it should be determined whether the manipulations of the ova inherent in Chambers' methods of examination have not produced abnormal behavior, which is of course of quite extraordinary interest in itself.

to constituent parts of the egg up to the time when they can no longer be separately distinguished. There is no great difficulty in following the nuclear derivatives of the spermatozoön; great uniformity is found in their composition and behavior throughout the entire plant and animal kingdoms. It is quite otherwise with the cytoplasmic derivatives of the spermatozoön which not only differ greatly in their composition, as concerns those parts admitted to the egg, but are also exceedingly difficult to follow. We shall consider first the behavior of the spermatozoön immediately after its entrance, and shall then discuss separately the nuclear and cytoplasmic constituents.

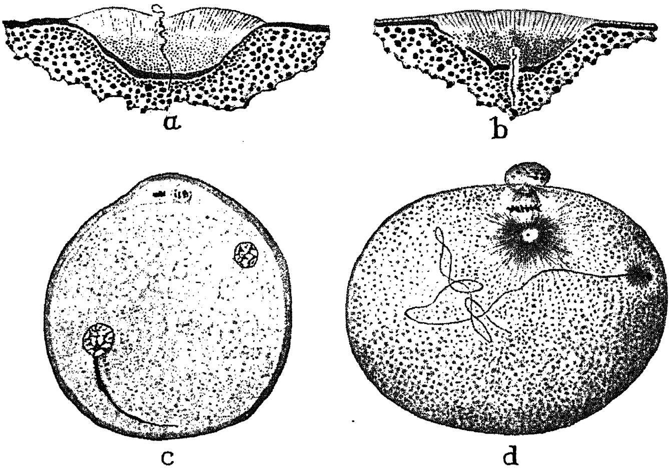

FIG. 6.—(*a*, *b*) penetration of the spermatozoön in the oligochaete *Rhynchelmis* (after Vejdovsky and Mrázek). Note the extensive yolk-free cone produced in the egg cytoplasm. (*c*) Spermatozoön in the egg of the bat *Vespertilio noctula* (after Van der Stricht). The entire spermatozoön enters. (*d*) The spermatozoön in the egg of the snail *Physa fontinalis* (after Kostanecki and Wierzejsky). The long, coiled tail of the spermatozoön lies in the egg cytoplasm; sperm centrosomes with aster between tail and head.

A. EARLY BEHAVIOR OF THE SPERMATOZOÖN WITHIN THE EGG

Immediately after penetration the head of the spermatozoön rotates around its own transverse axis through 180° (Figs. 7 and 8), so that the base which was external at the time of entrance becomes turned toward the center of the egg (cf. Henking, 1891; Wilson, 1895, 1896; Boveri, 1898, 1901; Meves, 1912, 1914; Lillie, 1912, and others). This phenomenon is very general and it may be universal; no adequate explanation has been found for it, and its significance is quite obscure. It seems to imply a polarization of the sperm nucleus with reference to the center of the egg.

This rotation is associated in the majority of the best-studied forms with the appearance of a sperm aster at the base of the sperm head (cf. Figs. 6, 7, 8). This aster appears during rotation, in the cases of *Toxopneustes* and *Nereis*, for instance; shortly after its origin a minute centrosome can be distinguished in its center (Figs. 7*e*, 8*b*). The centrosome and aster thereupon divide and produce a sperm amphiaster, which in some cases, at least, becomes the amphiaster of the first segmentation spindle (Figs. 7 and 8). It should be emphasized that in certain species of which *Ascaris* and mammals are striking examples (cf. also Wheeler, 1895, on *Myzostoma*) a sperm aster is not formed in the early internal stages of fertilization.

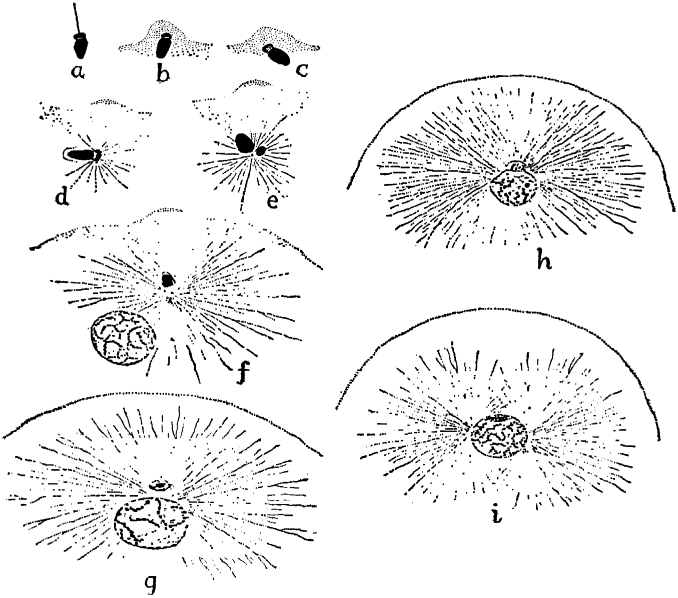

FIG. 7.—Fertilization of the egg of the sea urchin *Toxopneustes* (after E. B. Wilson): (*a*) the spermatozoön; (*b*, *c*) the sperm head and middle piece immediately after entrance; tail apparently absent; beginning of rotation *c*; (*d*) rotation halfway completed; origin of sperm aster; (*e*) rotation completed, middle piece separated from sperm centrosome; (*f*, *g*) approach of the germ nuclei; growth of the sperm aster; (*h*) meeting of the germ nuclei; division of the sperm aster; (*i*) first segmentation nucleus in which the sperm and egg components are readily distinguished.

The fact that the sperm aster is centered at the base of the sperm head in all cases in which its actual beginning has been traced led early to the supposition (cf. Boveri, 1887, 1901; Wilson, 1895, 1896, 1900), that its origin is due to

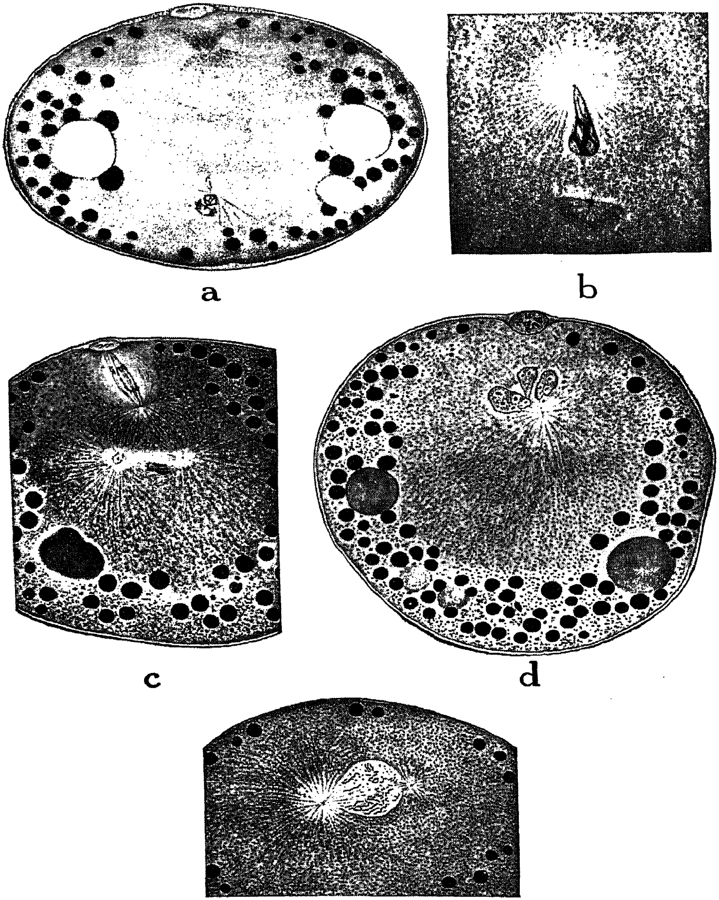

FIG. 8.—Sections of successive stages showing the internal phenomena of fertilization in *Nereis*. (*a*) Section of an egg 54 minutes after insemination. The head of the spermatozoön has rotated (cf. Fig. 5), the sperm nucleus is becoming rounded, and the sperm aster is beginning to arise opposite to the cone. The latter marks the apex of the sperm head. The first polar body is fully formed. (*b*) The sperm cone-nucleus-aster complex of an egg 64 minutes after insemination. The sperm cone is now separated from the nucleus, and is destined soon to disappear. The sperm aster and centrosome better developed. (*c*) 67 minutes after insemination; stage of anaphase of second maturation division. The sperm aster has divided, forming an amphiaster. (*d*) 77 minutes after insemination. The sperm nucleus lies to the left below; the sperm amphiaster has become reduced. The egg nucleus, which is formed by fusion of chromosome vesicles, is represented by two still unfused parts to the right above.

action of the middle piece of the spermatozoön. The sperm centrosome, therefore, which appears so soon after the aster becomes visible was regarded as a morphological (Boveri) or possibly chemical (Wilson, 1900) derivative of the middle piece or part thereof; and this determination was then correlated with the observation that in the histogenesis of the spermatozoön the centrosome (or part of the centrosome) of the spermatid becomes located in the middle piece. It was therefore concluded, more especially by Boveri (1899), that the centrosome of the sperm aster within the egg is derived from the centrosome of the spermatid. Inasmuch as it was further shown, as noted beyond, that in certain species the sperm amphiaster forms the first segmentation spindle, Boveri was able to furnish a plausible basis for his familiar theory of fertilization (cf. p. 520), viz., that the spermatozoön introduces an active division center (centrosome) into the egg (which has lost its center) and that initiation of development is due to this cause. This conception involves a theory of cell division in terms of the centrosome, which has historical interest certainly, but which appears inconsistent with current cellular physiology.

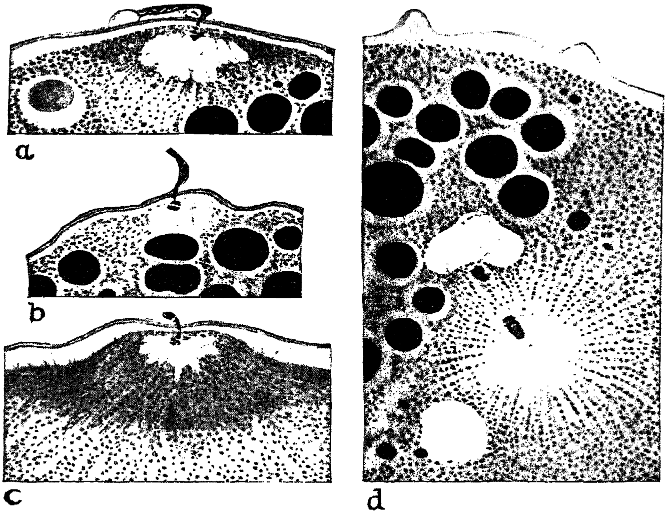

FIG. 9.—Effects of centrifugal force on penetration of the spermatozoön in *Nereis:* *a*, *b*, and *c* show removal of varying portions of the sperm head before penetration; *d* shows the later history of an egg into which a minute portion of the spermatozoön has entered; this part has produced a sperm aster and centrosome, although it represents only a fraction of the apical end of the sperm head similar to the piece shown in *c*.

So far as the theory of fertilization is concerned the demonstration of Boveri's theory would demand at the very least the proof of the identity of the centrosome of the sperm aster with the centrosome of the spermatid; and this proof is conspicuously lacking. In the case of the sea urchin, the sperm aster is centered not around the middle piece but to one side of it (cf. Fig. 7*e*), a determination that has been confirmed in a very detailed way by Meves (1912, 1914). Moreover, a differentiated centrosome is not demonstrable in the center of the sperm aster in its early stages, and never in association with the sperm nucleus prior to the appearance of the aster (cf. Boveri, 1895, 1901). In *Nereis* the middle piece of the spermatozoön, usually supposed to contain the spermatid centrosome, does not even enter the egg; nevertheless a sperm aster arises at the base of the sperm head. Moreover, Lillie (1912) has shown that it is possible by application of a strong centrifugal force to the inseminated eggs of *Nereis* to remove variable portions of the base of the sperm head itself before penetration, and that the remaining apical fragments of the sperm nucleus that enter the egg nevertheless cause formation of sperm asters at the base of the fragment in the usual way (Fig. 9). It is therefore experimentally demonstrated that the sperm aster does not owe its origin to a centrosome introduced into the egg by the middle piece or base of the head of the spermatozoön.[1] The physiological interpretation of the sperm nucleus-aster complex will be considered beyond.

B. MOVEMENTS OF THE GERM NUCLEI; THE FIRST SEGMENTATION NUCLEUS

The nucleus derived from the head of the spermatozoön is known as the sperm nucleus or male pronucleus.[2] It is destined to unite with the egg nucleus or female pronucleus derived from the internal daughter-chromosome group of the second maturation division of the egg to form the first segmentation nucleus or first nucleus of the zygote. The chromatin of the sperm nucleus is in its most condensed condition at the time of entrance; shortly thereafter the sperm nucleus begins to enlarge by imbibition of fluid (cf. Fig. 5), and tends to become vesicular. This change, however, proceeds relatively slowly during the maturation of the egg (cf. Fig. 8*a* and *b*). After the second polar globule is formed, the sperm nucleus and the egg nucleus enlarge at practically the same rate, and gradually approach the karyokinetic center of the egg where they come into contact. They are then usually of the same size and appearance (Fig. 10), so that they can be distinguished only by their positions or associations.

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[1] Recent results appear to destroy the possibility of any modification of Boveri's theory, even the assumption that the spermatozoön introduces an unorganized substance that is responsible as such for the formation of the sperm aster. The spermatozoön does not cause the formation of asters within endoplasmic spheres deprived of cortical material (Chambers, 1921; Just, 1923*a*; see p. 479), although all the postulated conditions of any modification of Boveri's theory are there present. As expressed by Lillie (1914, 1919), the spermatozoön needs itself to be fertilized, a process that is apparently effected in the cortex of the egg. Fertilization is, indeed, a mutual process that affects both partners concerned; it is not exclusively an effect of the spermatozoön upon the egg. This is of course most clearly seen in the conjugation of *Ciliata*, but the mutual effect is never lost in the course of evolution.

[2] The term "pronucleus" was first introduced by Van Beneden (1875) to indicate the substances derived from the spermatozoön and the egg respectively out of which he believed the first nucleus of the embryo was formed. As he was uncertain of the nuclear origin of these substances he designated their formations *pronuclei*, i.e., as destined to form a nucleus by their union, but not certainly to be recognized as nuclei themselves. On account of the prejudicial etymology the terms male and female pronucleus should be dropped.

However, when maturation of the egg is completed before fertilization, as in the sea urchin, the egg nucleus and the sperm nucleus proceed directly to the place of meeting, and the sperm nucleus is much smaller and more condensed than the egg nucleus at the time of union (Fig. 7*g*, *h*, *i*). If the meeting of the nuclei is prevented by the use of anaesthetics, as in Wilson's experiments (1901), the sperm nucleus may enlarge to the size of the egg nucleus, and, after recovery from the anaesthetic effect, the two equal nuclei unite.

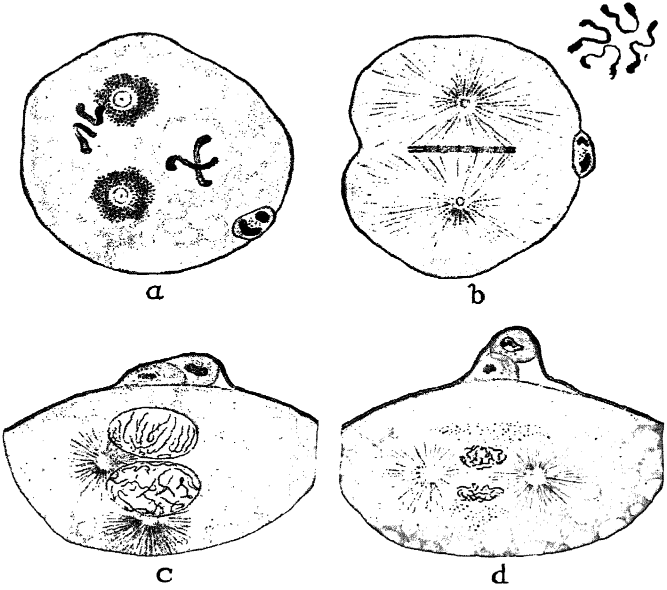

FIG. 10.—(After Boveri): (*a*) Germ nuclei of *Ascaris megalocephala*, approaching between the attraction spheres of the first cleavage spindle, each containing two chromosomes. (*b*) The first cleavage spindle fully formed; it contains four chromosomes which are shown in a polar view of the same spindle in the small figure to the right above. Two of these chromosomes are of maternal and two of paternal origin. (*c*) Meeting of germ nuclei of *Pterotrachea* (pteropod); each contains sixteen chromosomes. (*d*) Formation of the first cleavage spindle in *Pterotrachea*. The maternal and paternal chromosome groups are separate.

We have, therefore, to consider two questions to the extent that morphological observation admits: (*a*) What determines the movements of the germ nuclei within the egg and their union? (*b*) What is the nature of the union quantitatively and qualitatively?

a) As regards the first question, Roux, in 1887, resolved the movements of the sperm nucleus within the egg into two components, which he called the penetration path and the copulation path. His observations were made on the frog's egg, in which the spermatozoön leaves behind it a trail of pigment, marking out its path, which is usually curved or exhibits an angle. He conceived the first part of the path to be a continuation of the direction of penetration; the second part of the path he conceived to be determined by an attraction between the egg nucleus and the sperm nucleus.

That there is an energy of penetration of the spermatozoön which persists in the same direction after entrance into the egg is scarcely tenable, because the penetration itself is not a result of the locomotor energy of the spermatozoön; there is also no reason to assume that the nuclei as such exert attraction on one another. Such an assumption has no basis in fact beyond the actual meeting of the germ nuclei, which can equally well be explained on other more general and reasonable grounds. The view has also been presented that the movements of the sperm nucleus are brought about by the sperm aster, the fibers of which were supposed to act as contractile elements. (Cf. Kostanecki and Wierzejsky, 1896.)

The movements of the germ nuclei within the egg depend on conditions of equilibrium of the various cell constituents which constitute a definitely ordered stream of events. The localization of the nucleus within the cell is in general always determinate. We have therefore to conceive that, as both sperm nucleus and egg nucleus are in physiological relations to the same mass of cytoplasm, which is preparing to divide, they must reach the same position of equilibrium within the cell, and hence of necessity meet. Their coming together is due, not to mutual attraction, but to independent movements toward the same part of the developing egg. This tendency cannot, however, manifest itself until after maturation is completed; hence the movements of the sperm nucleus prior to the completion of maturation are not always directed toward the ultimate place of union of the germ nuclei, being under the influence of a different condition of equilibrium of the egg cytoplasm. The curved or bent path of the spermatozoön in certain cases follows from this, and it is not found in the echinids, where maturation is complete before fertilization.

C. ORIGIN OF THE CLEAVAGE CENTERS AND THE FIRST SEGMENTATION SPINDLE

In some animals (echinids, annelids, some Mollusca, ascidians, etc.), the sperm amphiaster becomes the achromatic part of the first cleavage spindle. In echinids the central body of the sperm aster divides and forms two, which move apart with consequent formation of an amphiaster, as the germ nuclei are on the point of meeting (Fig. 7*g*, *h*, *i*). The plane of separation in this case is at right angles to the line uniting the centers of the two germ nuclei, the asters thus pass to opposite sides of the segmentation nucleus, oriented definitely with reference to the sperm and egg components. The karyokinetic figure arises in the usual way between these asters, and the sperm and egg chromosomes therefore occupy opposite sides of the spindle. In those cases in which the egg has part of the maturation process still to complete, the sperm amphiaster remains more or less quiescent during maturation (Fig. 8); but it may entirely disappear (*Unio*, Lillie, 1895), in which case the asters and presumably the centrosomes also of the first cleavage spindle are new formations; or it may diminish to a variable extent (cf. *Nereis*, Fig. 8*c*, *d*, *e*). In other cases (e.g., *Ascaris*), the centers of the first cleavage spindle do not appear until about the time of meeting of the germ nuclei; in this case Boveri supposed them to be derived from a sperm centrosome included in the "archoplasm" accompanying the sperm nucleus. In the case of *Crepidula*, according to Conklin (1894, 1901*a*, 1904), one of the centers of the first cleavage spindle arises in close association with the sperm nucleus, the other with the egg nucleus.

In any case, the orientation of the first cleavage spindle is always symmetrical to the germ nuclei and at right angles to the line uniting them so that maternal and paternal chromosomes lie on opposite sides of the spindle (cf. Fig. 10).

The morphological variations are very numerous with reference to the origin of the first cleavage amphiaster when we compare all the various species studied.[1] In our opinion, these variations do not belong properly to the subject of fertilization as such, but rather to the subject of karyokinesis. The physiological reactions must be presumed to be uniform; the morphological variants may therefore be attributed to time differences in the components of the physiological reactions in different species dependent upon time of entrance of the spermatozoön and the biophysical and biochemical composition of the protoplasm.

D. THE PHYSICAL BASIS OF HEREDITY

If heredity is to be understood in its broadest sense as repetition of individual development, then undoubtedly nothing less than the entire zygote can be regarded as constituting its physical basis; but if the word heredity is to be understood in the usual technical sense as study of the behavior of differentiating characteristics of parental and filial generations, we have to ask what parts of the gametes are more particularly concerned in the known genetic

[1] An interesting episode in this history was furnished by Fol (1891), who described both a sperm centrosome and an egg centrosome in the sea-urchin egg each of which divided in two about the time of meeting of the germ nuclei, the daughter-halves then separating and migrating around the nucleus, until meeting and union of pairs derived from sperm and egg centrosomes ensued. To this phenomenon he gave the name "quadrille of the centers." After two or three rather hasty confirmations of the phenomenon in other forms, the entire series of observations was disproved or abandoned.

laws governing these phenomena. The following discussion is based on the technical usage. It is obvious that the study of the relations of the parts of the gametes in the composition of the zygote must furnish the foundation of any theory of heredity in this sense. We shall consider, therefore, first the germ nuclei and their chromosomes, and second the cytoplasmic constituents of the gametes.

a) THE GERM NUCLEI AND THE CHROMOSOMES

We have seen that the germ nuclei may apparently fuse together to form a single nucleus called the first segmentation nucleus (e.g., echinids, teleosts, mammals). But in many, perhaps most, cases the changes preparatory to the first cleavage of the egg begin before such a fusion occurs, and in these cases it is easy to determine that each germ nucleus contributes the same number of chromosomes to the first segmentation spindle (Fig. 10). Even in those cases in which a typical first segmentation nucleus occurs (Fig. 7*i*) it is equally certain that the maternal and paternal chromatins form equal chromosome groups upon the first segmentation spindle; they can in fact usually be distinguished; moreover it is known that each germ nucleus contains only the haploid number of chromosomes, whereas the first segmentation spindle has always the diploid number. In other words, it is demonstrated that there is no fusion of maternal and paternal chromosome groups, and that each group maintains its independent existence on the first cleavage spindle. No other nuclear constituents can be traced beyond this stage.

Van Beneden (1883) was the first to discover this invariable law of fertilization in the case of *Ascaris megalocephala*, where each germ nucleus produces two chromosomes, so that the first segmentation spindle contains four (Fig. 10*a*, *b*). As these divide longitudinally in the usual way, each of the first two cells receives four daughter-chromosomes, two of maternal origin and two of paternal origin. Van Beneden assumed that this condition was transmitted to all subsequent generations of cells, and that therefore all nuclei of the individual are of biparental origin, a conception that explains the equal effects of the parents in heredity, and the intimate intermingling of parental characteristics in the offspring.

The conception of the equal biparental character of nuclei, which we owe originally to Van Beneden, has received abundant cytological and genetic proof, the consideration of which would carry us far beyond the bounds of this section. We should, however, allude to Moenkhaus' (1904) interesting observations on hybrid fish in which he could follow maternal and paternal chromosome groups differentiated by morphological characters into late cleavage stages (cf. also Herla, 1894; Zoja, 1895; and numerous later studies on hybrid chromosome groups).[1]

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[1] Reference should also be made to the arguments for biparental composition of the nuclei derived from the double nature of the cleavage nuclei in certain forms (cf. Haecker, 1902, 1904; Rückert, 1895; Conklin, 1901).

Undoubtedly the most fundamental fact which the morphological study of fertilization has revealed is the separate persistence of the germ nuclei and their equivalence with reference to the chromosomes. In all other respects the germ cells are differentiated in opposite directions. This determination furnishes the foundation for all chromosome theories of heredity from the time of Weismann down to the present day.

The morphological and genetic equivalence is also physiological in the sense that either germ nucleus is adequate by itself for purposes of development. This is shown for the egg nucleus by artificial parthenogenesis, and for the sperm nucleus by those experiments in which an enucleated fragment of an egg fertilized by a single spermatozoön has been proved to develop (*Merogony*). Boveri (1889) attempted to show by experiments that the inheritance in hybrid merogony was purely paternal, i.e., determined alone by the nucleus derived from the father and unaffected by the cytoplasm derived from the mother. However, the differentiating characters of the larvae of the species used were not adequate for a conclusive demonstration (cf. Seeliger, 1894, 1896; Morgan, 1895). The demonstration that Boveri attempted was furnished later by analysis of gynandromorphs in which a purely paternal inheritance has been

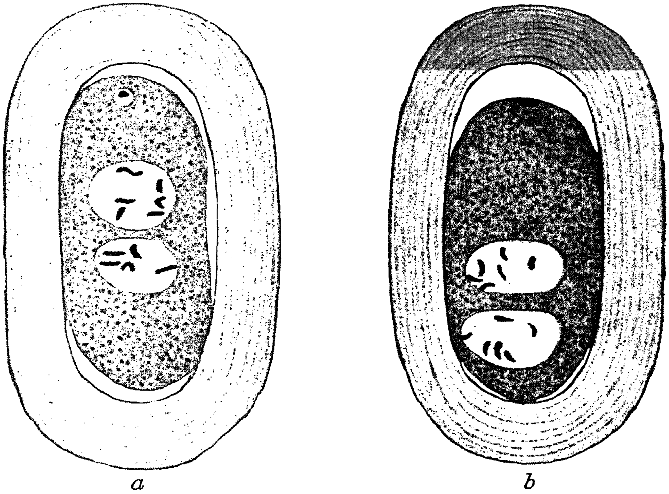

FIG. 11.—Fertilization of a nematode (*Ancyracanthus cystidicola*) (after Mulsow): In each figure the upper nucleus is the egg nucleus, the lower the sperm nucleus. In both figures the egg nucleus contains six chromosomes; in *a* the sperm nucleus contains five chromosomes, in *b* six. The combination 6+5 in *a* gives the male number, eleven; the combination 6+6 in *b* gives the female number, twelve. The two classes of spermatozoa are hence regarded as male-producing and female-producing, respectively.

demonstrated for those portions of the body possessing haploid nuclei of male origin (cf. Morgan and Bridges, 1919).

The only differences that have been shown to occur on the morphological side between the maternal and paternal chromosome complexes are those related to sex determination (Morrill, 1910; Mulsow, 1912; see Fig. 11). Such differences support the chromosome theory of heredity. Differences in regard to genes are of course indecipherable morphologically. The foundation of all genetic theory of sexually produced organisms rests upon the demonstrated chromosomal equivalence of the germ nuclei.

b) THE RÔLE OF THE CYTOPLASM

The spermatozoön usually introduces certain cytoplasmic constituents into the egg, but as contrasted with the nucleus the history of such parts cannot usually be followed very far. In some cases, as in *Ascaris*, the quantity of cytoplasm thus introduced is very considerable (Fig. 3); in other cases none can be demonstrated (*Nereis*, Figs. 5 and 8). It is generally believed with good reason that the perforatorium and tail have no further significance in fertilization than to aid in the union of the gametes. In any event they are not traceable after a very early state. Attention has been focused, largely on theoretical grounds, on two constituents usually forming part of the middle piece of the spermatozoön, viz., the centrosome and the mitochondria derived from the spermatid.

The problem of the centrosome has already been discussed (pp. 461-64); not even Boveri maintained that it played a part in heredity, but he regarded it essentially as an activating agent.

The other cytoplasmic element that has been claimed to play an important rôle in fertilization is the mitochondria (called plastochondria by Meves), which form such an apparently important constituent of all classes of cells. The special protagonist of the significance of this substance in fertilization is Meves, who maintains that it is concerned in the transmission of hereditary characteristics, basing this view on the part that he believes it to play in protoplasmic differentiation. He found an apparently very demonstrative case in *Ascaris megalocephala* (Meves, 1911), the spermatozoa of which contain large numbers of mitochondrial granules in the large cytoplasmic body surrounding the nucleus (cf. Fig. 3). The entire spermatozoön penetrates in this case, and the mitochondrial granules of the sperm cytoplasm continue to surround the nucleus long after penetration; by degrees they become intermingled with the mitochondria of the egg, and Meves even hazarded the conjecture that they possibly united by pairing with the mitochondrial granules of the egg; he therefore considered his view that the mitochondria play an important rôle in heredity justified.

Pursuing the matter farther, Meves (1912 and 1914) made an exceedingly careful study of the fate of the middle piece of the spermatozoön in the fertilized eggs of echinids. He found that this minute fragment could be traced intact into one of the first two cells; in successive cell divisions it always passes intact into one of the daughter-cells only, and may be found in the eight-celled stage either in one of the animal or one of the vegetative quartet; he even traced it to the thirty-two-celled stage. Thus it is not broken up and distributed to all of the cells, nor yet transmitted constantly to any one part of the egg, as the theory that it represents a substratum for bearing heredity factors would require; neither does it exhibit any signs of activity. In the egg of an ascidian (*Phallusia*) the same author (1913) could follow the sperm mitochondria through part of the fertilization stages, but then lost sight of them. Van der Stricht (1902), in the bat, and Lams, in the guinea pig, found that the tail of the spermatozoön and connecting piece which carries mitochondria pass into one only of the first two cells. Finally, in *Nereis*, according to Lillie (1912), and in *Platynereis*, according to Just (1915*c*), the middle piece of the spermatozoön, which is usually supposed to carry the mitochondria, does not enter the egg at all.

Whatever may be the function of the mitochondria in cell physiology, it must be admitted that the study of fertilization has shown no reason for the assumption that their introduction into the egg by the sperm in certain species is concerned in the transmission of paternal characteristics. The variable quantity in different cases and the distribution to single blastomeres in certain cases exclude the hypothesis that they have any specific paternal hereditary effect. There is no reason to deny that sperm mitochondria function in the egg when present, but if so it is probable that they are not differentiated in their chemical composition or genetic behavior from the mitochondria of the egg itself.

The egg cytoplasm.—The egg cytoplasm and its inclusions constitute an exclusively maternal material which determines many of the characters of early embryonic stages; such characters are therefore exclusively maternal. The materials of the cytoplasm are, however, being constantly consumed in the metabolism, and the process of renewal and increase of such materials involves interaction of nucleus and cytoplasm; therefore the purely maternal cytoplasm soon disappears, and is replaced by cytoplasm formed under the influence of the biparental zygote nucleus. Maternal cytoplasmic characters cannot, therefore, survive long in the life-history, unless the cytoplasm contains elements either that survive as such or that increase independently of the nucleus. In plants plastids, including the chlorophyll grains of chloroplasts, have a history of this sort. There may be conceivably similar elements in animals that have a purely cytoplasmic history, but for this we have little evidence. We have, however, adequate cytological grounds in the possible occurrence of such persistent elements of composition of the egg cytoplasm as basis for the explanation of the rather rare cases of exclusively maternal inheritance from a zygote known to the geneticists. Purely paternal inheritance probably does not exist in any regularly formed zygote, and this constitutes an independent line of negative evidence against cytoplasmic inheritance from the male side.

4. *Polyspermy:*

The ova so far considered are normally monospermic; there are, however, certain ova into which more than one spermatozoön enters normally, and practically all ova may become polyspermic under abnormal conditions. We may thus distinguish normal, or physiological, and pathological polyspermy.

A. PHYSIOLOGICAL POLYSPERMY

Physiological polyspermy occurs in vertebrates possessing eggs of large size devoid of a strong membrane and micropyle, in which penetration of the spermatozoön may occur at any spot within a large area. We may conceive in such cases that the protective mechanism against penetration of supernumerary spermatozoa, which begins to form at the point of penetration and spreads, does not extend itself with sufficient rapidity to protect the entire fertilizable surface from other spermatozoa. The large eggs of sharks, of some Amphibia, of reptiles, and of birds are thus polyspermic. Polyspermy occurs in the eggs of several classes of insects which possess several micropyles (Henking, 1891). Among animals possessing small eggs, it occurs apparently only in Bryozoa, in which the spermatozoa are united in bundles (Bonnevie, 1907).

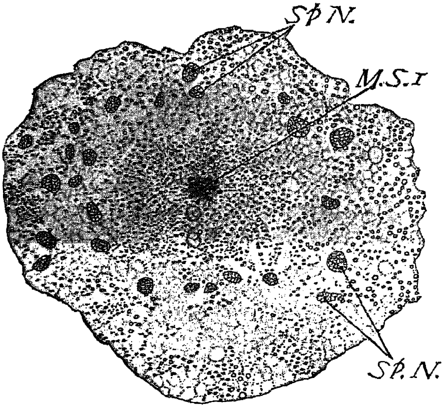

FIG. 12.—Part of a horizontal section of the germinal disk of a pigeon's egg freed from the ovary, but not yet in the oviduct. First maturation spindle (*M.S. 1*) cut transversely in the center; sperm nuclei (*Sp. N.*) surrounding it (after E. H. Harper).

In all cases of normal polyspermy only one of the sperm nuclei formed from the entering sperm heads unites with the egg nucleus, and the supernumerary sperm nuclei are disposed of in certain ways. Thus the fertilization in such cases is finally monospermic. In the fertilization of the pigeon, for instance, from about twelve to twenty-five spermatozoa enter the germinal disk as soon as the ovum is released from the ovary (Harper, 1904). The second maturation division occurs after this, and during this time the sperm heads accumulate in a ring of protoplasm surrounding the maturation spindle at some distance from it (Fig. 12). After completion of this division and formation of the egg nucleus, one of the sperm nuclei moves centrally and unites in the usual way with the egg nucleus, while the supernumerary sperm nuclei move away from the center as though repelled and accumulate in the peripheral periblast. Here they undergo division and produce cell areas in the periblast, which are a conspicuous feature of the development up to about the thirty-two-celled stage, at which time, according to Miss Blount (1909), they begin to degenerate, and soon entirely disappear. The segmentation nucleus in this case is thus formed of the union of the egg nucleus and a single sperm nucleus in the usual way, and all nuclei of the embryo are derived from this by karyokinetic division.

There would thus seem to be no advantage connected with physiological polyspermy; at the most it is harmless, and merely represents a condition in which the final determination of the successful spermatozoön is completed within the egg. Such eggs have in some way overcome the usually harmful effects of polyspermy. Bonnevie (1907), however, is of the opinion that in Bryozoa at least it is significant for the maintenance of the organism; she suggests that the supernumerary spermatozoa furnish extra-nuclear chromatin of physiological importance. There is, however, no adequate foundation for such a view at present.

B. PATHOLOGICAL POLYSPERMY

Eggs normally monospermic may be entered by more than one spermatozoön if they are allowed to become stale before insemination; the same result may be attained by exposing them to the action of various injurious substances, such as chloroform, chloral hydrate, cocaine, nicotine, strychnine, quinine, and many others, in appropriate concentrations for proper periods of time. Moreover, a very heavy insemination of any normal lot of eggs will usually yield a small percentage of polyspermy. According to Smith and Clowes (1924), polyspermy occurs in nearly every egg at a definite hydrogen-ion concentration of the sea water (see Fig. 17) even with an ordinary light insemination. The number of spermatozoa that may enter under various abnormal circumstances may range from two to a considerable number. The first student of this subject, Fol, in 1877, determined for the starfish and sea urchin that polyspermic eggs divide in more than two cells at the first cleavage and their subsequent development is never normal.

In 1887 O. and R. Hertwig published a detailed study of polyspermy in the sea urchin; each sperm nucleus forms an aster which subsequently divides to form an amphiaster. If only two sperm nuclei are present both unite with the egg nucleus, and the two amphiasters produce a four-poled karyokinetic figure, or tetraster; the egg divides simultaneously into four cells, but the subsequent division of the cells is always in two each. Triasters sometimes form, owing to fusion of two asters, and a simultaneous division of the egg into three cells follows. If more spermatozoa enter, all sperm nuclei do not necessarily unite with the egg nucleus; two or more may unite with the egg nucleus and a multipolar figure results; the other sperm amphiasters then associate themselves with this figure and very complex karyokinetic systems result.

In the frog (Herlant, 1911), each sperm nucleus forms an aster as in the sea urchin, but the egg nucleus unites with only one of the sperm nuclei; the supernumerary sperm nuclei form karyokinetic figures also. Thus in the case of dispermy two karyokinetic figures result, one of which contains the chromosomes of the egg and one sperm nucleus, the other only the chromosomes of the supernumerary sperm nucleus. In the case of trispermy we have three karyokinetic figures—one diploid, two haploid. The dispermic egg divides in two cells and the trispermic in three, but each of these cells is binucleated. In the dispermic egg one nucleus of each cell is diploid, the other haploid; in trispermic eggs this applies to two of the cells, but the two nuclei of the third cell are both haploid. In subsequent divisions the proportion of diploid nuclei is maintained.

In the sea urchin, in the frog, and also in all other cases so far as known, pathological polyspermic eggs produce abnormal embryos, which soon die. Boveri (1902, 1907) has made a most careful and interesting analysis of conditions in the sea urchin, which led him to the conclusion that the ill effects are due in this case to abnormal distribution of the chromosomes. Taking the simplest case of dispermy he shows that the distribution of chromosomes in the tetraster is highly irregular and a matter of chance, from which it results that the four nuclei formed have different numbers of chromosomes. This would not in itself account for the abnormal results, because it is known that half the diploid chromosome number is sufficient for normal development, and he could show that the number in each nucleus exceeds this number on the average. From this he argues that the *chromosome composition* of the nuclei must be on the average inadequate; that not merely a given number of chromosomes, but a definite qualitative composition of the chromosome group, is necessary for normal development. He thus conceives that the chromosomes of each germ nucleus are qualitatively differentiated, and that a full representation of chromosome qualities is necessary for normal development. His experiments constitute an argument for qualitative differences of chromosomes which has been generally accepted. The result is reached by exclusion of other possible causes of abnormality. This subject leads into certain phases of cytology that do not belong in our field.

The case of the frog is somewhat different, in that the nuclei are either definitely diploid or haploid. Herlant comes to the conclusion that the cause of death in this case is the different size of the nuclei and their associated cell bodies in the same embryo, which renders normal functioning impossible, and other more obscure probable causes of disharmony associated with this principle.

For the subject of morphology of fertilization the study of polyspermy is significant in two principal respects: (1) It furnishes the demonstration that the sperm nucleus is different from the egg nucleus, owing either to association of a centrosome with it or for other cause; because we find that each sperm nucleus produces a definite effect on the cytoplasm of the egg, the formation of an aster, which the egg nucleus itself does not produce in the cases studied. (2) The inevitable pathological result, when more than one sperm nucleus is concerned in the development, furnishes important evidence for the nuclear theory of heredity. On the physiological side the study of polyspermy is significant from other aspects, which we shall examine later.

II. THE PHYSIOLOGY OF FERTILIZATION

Fertilization involves a series of physical, chemical, and unanalyzed biological reactions. The series as such is irreversible, and fertilization can, therefore, not be repeated, nor can it be superimposed upon parthenogenesis. In this particular, fertilization resembles progressive differentiation and is unlike the reversible functioning of differentiated cells. Fertilization may be partial in one of two senses, either that the series of reactions may be arrested at various stages, or that the reactions may be quantitatively deficient;[1] the study of partial fertilization offers important data for analysis.

The fertilization reactions are both tissue-specific and to a great extent species-specific. In the latter case the initial, cortical, events of fertilization present a higher degree of specificity than the later events of the series, so that when the cortical resistance against hybridization is broken down, as it may be by the use of various reagents, the internal events of hybrid fertilization may proceed quite normally to varying degrees in different cases (see p. 509).

In order to have a correct point of view for considering the phenomena of the physiology of fertilization it is necessary to bear in mind that the egg is in a real sense a self-activating system, or at least a system that can be activated by other agents than the spermatozoön, as in cases of parthenogenesis. The activation may thus be studied by itself; but such studies must not be designated "chemical fertilization," however useful they may be in analyzing certain problems of fertilization. We must similarly guard against regarding penetration of the spermatozoön as synonymous with fertilization, for, on the one hand, we may have fertilization well begun (as in *Nereis*) before entrance of the spermatozoön into the egg, and, on the other hand, we may have penetration of the spermatozoa into the egg without any fertilization if the egg is not in the proper condition. The penetration of the spermatozoön is one of the problems of fertilization, but it is not the whole problem.

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[1] There is a third sense in which we may speak of partial fertilization, viz., the original sense of Boveri (1888*b* and 1915), when the egg nucleus divides without union of the sperm nucleus, if the latter then fuses with one of the daughter-nuclei. This would result in an individual, one half of which possessed haploid and the other half diploid nuclei; or, if the union of the sperm nucleus were delayed until later divisions of egg nuclei, smaller parts would be diploid and larger parts haploid. This form of partial fertilization has been theoretically postulated to account for certain insect gynandromorphs (cf. Morgan, 1905, and Boveri 1915). In the sense of activation, however, the entire egg is fertilized in such a case, so that it need not come up for further consideration in the physiological part of the present section.

1. *Conditions of the gametes necessary for fertilization:*

The fertilization reactions are possible only during a limited period of the life of the gametes. This we may call the fertilizable period. Knowledge of the conditions of the gametes that fit them for entering into the fertilization reactions should contribute to an understanding of the physiology of fertilization.

A. THE SPERMATOZOÖN

Spermatozoa attain fertilizing power after the completion of histogenesis and the attainment of full motility; unripe spermatozoa will not fertilize. It is usually assumed that ripe spermatozoa retain fertilizing capacity as long as their motility persists; but while it seems to be fully demonstrated that motility is necessary for fertilization, there are reasons for believing that loss of fertilizing power may occur before loss of motility. (See below.)

It seems probable that spermatozoa are incapable of receiving nourishment outside of the gonad or after they are fully differentiated. We must, therefore, regard these cells as charged with their full store of energy in the testis and their capacity for locomotion as thus limited. The duration of their strictly limited term of life is, therefore, a function of activity. Sperm suspensions retain their vitality for a relatively long period of time if activity is reduced or suppressed, and lose it relatively rapidly if activity is great. Cohn (1918) has determined that the total CO_2 production per unit sperm in suspensions is the same whether their life is long or short (sea urchins). As spermatozoa are soon inactivated by CO_2, the duration of life of spermatozoa and their fertilizing power are relatively long in concentrated suspensions, where activity is soon decreased by their own CO_2 production, as compared with dilute suspensions where the CO_2 production is not sufficient to suppress movement. (Cf. Cohn, 1918.) Similarly, the duration of fertilizing power of sperm suspensions may be prolonged also by other inactivating agents, such as KCN, that do not kill the spermatozoa.

Comparison of the duration of fertilizing power in sperm suspensions of sea urchins of graded dilutions have, however, shown that fertilizing power is lost before capacity for motility. Thus the fertilizing power of perfectly fresh sperm suspensions plotted with dilution as abscissae and percentage of eggs fertilized as ordinates gives the logarthmic curve *a*, while sperm suspensions twenty minutes old give the curve *b* (Fig. 13). Thus it is seen that while some fertilizing power exists with perfectly fresh sperm down to a dilution of $1/2^{25}$, fertilizing power is lost at about $1/2^{10}$ in suspensions that have stood twenty minutes. Experiments showed that sperm suspensions of $1/2^{17}$ decline to zero in fertilizing power in about six minutes, those of $1/2^{14}$ in about sixteen minutes, those of $1/2^{8}$ in about two hours, while 1 per cent sperm suspensions may retain fertilizing power for about two days.

The rate of loss of fertilizing power is much more rapid than loss of motility, a fact that is particularly noticeable at dilutions below about $1/2^{10}$. Thus in one experiment (Lillie, 1915), eggs were added to a $1/2^{10}$ sperm suspension that was on the point of complete loss of fertilizing power. The spermatozoa were active, and entered the jelly of the eggs to such an extent that in ten eggs selected at random an average of nine spermatozoa was counted in contact with each egg in an optical section; the eggs, however, remained unfertilized. The idea suggested by Glaser (1914) that the spermatozoa exert a mass influence in fertilization, and that more than one is needed, seems to depend on observations of this kind where slightly stale sperm suspensions were used (cf. also Schücking, 1903).

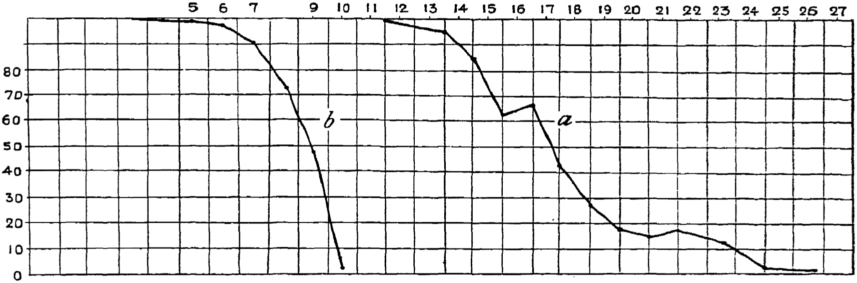

FIG. 13.—Logarithmic curves showing the fertilizing power of dilutions of sperm of *Arbacia*. The ordinates represent percentages of eggs fertilized; the abscissae dilutions of sperm in powers of 2. Curve *a* perfectly fresh sperm suspensions; curve *b* sperm suspensions 20 minutes old.

Such loss of fertilizing power before loss of motility suggests the idea that the spermatozoön carries a fertilizing substance which may be lost. The conception of such a substance is a very natural one, and various attempts have been made to isolate it from sperm extracts and to fertilize eggs (cf. Winkler, 1900; Gies, 1901; Loeb, 1913*a;* Robertson, 1912*a* and 1912*b;* Just, 1922*a*). The results of such experiments have invariably proved negative, whether for the reason suggested by Loeb that the postulated fertilizing substance requires the motive power of the spermatozoön to make it effective against the egg or for other reasons.[1]

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[1] Loeb (1913, pp. 201–6) has given an excellent review of this subject. He points out that all the alleged positive results are due either to presence of live sperm or to conditions, such as increased alkalinity, or hypertonicity of the sea water, that by themselves may produce parthenogenesis. Robertson (1912) by a very complicated method claimed to have extracted a substance from the spermatozoa of *Strongylocentrotus purpuratus* that acts as a powerful fertilizing, agglutinating, and cytolyzing agent upon the eggs of the same species, and which he identifies with a substance (oöcytin) obtained by similar methods from ox-blood

The main principle of this discussion, viz., that spermatozoa may lose their fertilizing power for other causes than loss of motility has obvious important bearings. The mere fact that spermatozoa may retain their motility for three weeks or more in the human genital tract (Waldeyer) by no means proves that they retain their fertilizing power during all this time, although this has been generally assumed. In an excellent paper published after his death, Mall (1918) points out the many contradictions and unnecessary assumptions that this belief entails with reference to the facts of human conception, and he concludes that it is probable that spermatozoa have lost their fertilizing power by the time they have passed the tube. Bryce and Teacher (1908) and Triepel (1914–15) also conclude that fertilization is limited to about a forty-eight-hour period after copulation.

B. THE OVUM

Ova attain a fertilizable condition rather suddenly. In certain cases, at least, as in *Dentalium*, *Cerebratulus*, and starfish, this is associated with the rupture of the germinal vesicle. O. and R. Hertwig (1887) showed that under the influence of chloral hydrate spermatozoa may penetrate unripe sea-urchin eggs in large numbers, but the eggs remain unchanged and the spermatozoa within the eggs likewise. Under normal conditions spermatozoa do not usually penetrate such eggs. Lillie has similarly observed penetration of unripe oöcytes of *Chaetopterus* without any subsequent reaction of egg or sperm. Wilson (1903) has observed in *Cerebratulus* (cf. also Yatsu, 1904, 1908) and Delage (1901*a*) in the starfish that enucleated fragments of full-grown eggs with intact germinal vesicle will not fertilize, but that shortly after the germinal vesicle has broken down similar fragments fertilize readily. Chambers (1921) has shown that various stages may be distinguished in the development of full capacity for fertilization after rupture of the germinal vesicle in the starfish egg; immediately after rupture the fertilization capacity is only partial, and gradually it becomes complete. Thus in these cases substances from the germinal vesicle bring about a protoplasmic maturation that is necessary for fertilization. In the case of *Nereis*, however, the egg reacts before rupture of the germinal vesicle (cf. p. 452), and in this case it may be supposed that the substances necessary for such protoplasmic maturation diffuse through the wall of the germinal vesicle.

Brachet (1922) finds that, after rupture of the germinal vesicle in the sea urchin, spermatozoa that penetrate eggs cause the formation of small centers

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serum. For the effective action of these substances in producing the appearance of membranes, previous sensitization of the eggs by $SrCl_2$ is necessary; when we consider the great variety of agents that produce surface changes in eggs, we may well require further proof that a specific fertilizing substance was actually demonstrated in the extracts. Just (1922*a*) has also studied this subject using oxalated sea water as medium for extraction, but he could not be sure that his positive results (on *Nereis*) were not due to agencies other than the sperm extract as such.

in the egg cytoplasm. But, as sea water inhibits the maturation process, the fertilization reactions do not proceed farther. In general in successively later stages of maturation the sperm-aster formation is more pronounced, but fertilization is never completed unless the egg is fully mature before penetration of the spermatozoön. Spermatozoa that enter eggs with intact germinal vesicle form no asters, nor do they undergo other changes. The sea urchin also, therefore, belongs with those forms that acquire capacity for fertilization immediately after rupture of the germinal vesicle; but on account of the inhibiting action of sea water on maturation the process does not go through to completion until maturation is completed.

When the fertilizable condition is attained the capacity for reaction is localized in the cortex. This idea is inherent in Lillie's theory of fertilization, and is concretely illustrated in Figure 1, page 579, of his paper on "The Mechanism of Fertilization in Arbacia" (1914). It is most clearly demonstrated by Chambers' proof (1921; cf. Lillie, 1919, p. 264), that fragments of fertilizable eggs of the starfish are fertilizable only if they possess some portion of the cortex of the egg; purely endoplasmic spheres, produced by tearing the cortex with the micro-dissection needle and allowing endoplasmic exovates to form, do not fertilize; although they may be entered by spermatozoa, the latter remain unaltered within the protoplasm. If even a small portion of the cortex remains on such a sphere the portion possessing cortical material forms a membrane, and the ability of the part to approximate normal segmentation is a function of the amount of the original egg cortex which it bears. There is thus a quantitative relationship expressed in terms of viability between cortical and endoplasmic material.

Just (1923*a*) has extended Chambers' determination of the necessity of the cortex for fertilization to *Echinarachnius*. Endoplasmic buds, which are readily produced, never give any signs of fertilization, while the remaining parts of the eggs from which they are derived fertilize readily and develop, and this without reference to the location of the egg nucleus either in the cortex-bearing part or endoplasmic bud. Essentially similar results have also been obtained by Hyman (1923).

These results show that the fertilizable condition of the ovum is due to modification of the cortex of the egg by a substance or substances given off from the germinal vesicle. They render it probable that the modification in question concerns the formation of a substance in the cortex of the egg that has special affinity for spermatozoa, or for some substance borne by them, and which also has an activating effect upon the ovum itself after suitable stimulation.

In some cases the fertilizable condition of the ovum is of very brief duration, and in any case it is a condition that may be lost before death of the egg. The most remarkable case of brief duration of the fertilizable condition is that of *Platynereis* described by Just (1915). Fertilization is normally internal in this annelid, and the eggs are laid immediately after. Eggs taken from the female can be artificially fertilized provided they are not first exposed to sea water, which may, however, be added without harm even five seconds after insemination. If, on the other hand, the eggs are placed in sea water for a few seconds before fertilization they become unfertilizable even if the sea water is filtered off. The effect may be graded by using minimal quantities of sea water, in which case partial fertilization may occur, the fertilized eggs exhibiting weakness and generally failing to segment. This condition resembles that described by Chambers for the starfish (see above), where he determined that the ability of a part to approximate normal segmentation is a function of the amount of the original cortex which it bears. The results in *Platynereis* permit only of one conclusion, viz., that the eggs lose in sea water a cortical substance necessary for fertilization.

The eggs of the wall-eyed pike (Reighard, 1893) rapidly lose capacity for fertilization in water; even after two minutes, more than half of the eggs fail to segment on fertilization; and all eggs lose fertilization capacity in ten minutes. Starfish eggs rapidly lose their capacity for fertilization after formation of the first polar globule (Delage, 1901). The eggs of the sea urchin will bear repeated washings in sea water without loss of fertilization capacity; but after a certain number of washings before insemination the developmental capacity becomes progressively reduced. This takes place much more rapidly if the jelly be first removed from the egg. This gelatinous covering acts apparently as protection against loss of a substance necessary for fertilization (cf. pp. 491, 492).

The capacity for parthenogenesis exhibits parallel relations to fertilization capacity. Delage (1901*a*) was the first to notice this in the case of the starfish egg. R. S. Lillie (1915) made a more detailed examination of this point with reference to heat parthenogenesis, and found that "warming before the dissolution of the germinal vesicle had begun was ineffective and in fact inhibited maturation entirely; the most favorable period lay between the breakdown of the germinal vesicle and the separation of the first polar body; after both polar bodies had separated development was imperfect and never proceeded far."

The inference that the failure to respond to parthenogenetic agents is due to loss of some substance in sea water was strongly supported by Just (1915*b*) in a study of heat parthenogenesis in *Nereis*. He found that if the eggs were first washed in sea water before warming they would not develop, or only an exceedingly small percentage were affected; if, however, the eggs without previous contact with sea water are exposed to a favorable elevated temperature for an optimum period of time (e.g., twenty-five minutes at 35° C.) in a small quantity of sea water, all of them may segment and as many as 20 per cent develop into trochophores.

C. DISCUSSION

The conditions of the gametes necessary for fertilization are shown by the preceding data to involve more than the mere continuation of vitality. In the case of the ovum it is clear, not only that the cortex is necessary for fertilization, but also that the cortex itself may lose this capacity during the life of the egg. The capacity for parthenogenesis runs parallel to that for fertilization, though apparently at a somewhat higher level. In the case of the spermatozoön, motility alone is not sufficient for fertilization. It seems impossible to think of these conditions in a purely physical sense, especially when we consider their capacity for minute gradation. If we think of them in a chemical sense it is necessary to postulate both a special substance borne by the spermatozoön and also one borne by the egg which interact in fertilization; the function of the egg-borne substance is expressed in all the processes of activation including the entrance of the spermatozoön and formation of the fertilization membrane. The spermatozoön thus does not act directly in activating the egg, but indirectly through a special substance in the cortex of the egg to which the name *fertilizin* has been given.

2. *Egg-secretions and their effects on spermatozoa:*

The ova of many species secrete substances of unknown chemical constitution into their natural medium, which may, however, be readily detected by their effects upon sperm suspensions of the same species. The results of study of these substances by sperm suspensions apply directly to analysis of the physiology of fertilization, for the substances detected by the spermatozoa can be shown to be derived from the cortex of the egg in which capacity for fertilization resides. If the eggs are laked in distilled water, or comminuted in sand or otherwise, substances from the endoplasm are also obtained, but the present considerations do not deal with such *extracts*, which are to be sharply distinguished from the normal *secretions* with which alone we are concerned. The egg secretions have been studied principally in echinids which are well suited to the purpose.

The secretions are found in the sea water in which unfertilized eggs have been standing; such sea water may therefore be briefly designated egg water. It of course contains CO_2 in addition to the more complex substances with which we are concerned. For the study of the latter the concentration of the sperm suspensions used as indicators should be at least one part of the sperm as it comes from the testes to ninty-nine parts sea water—briefly 1 per cent sperm; the reactions can be observed by placing some drops of the sperm suspension on a slide and covering it by a long cover slip supported by glass rods about 1 mm. in diameter; the egg water to be tested is then injected into the suspension through a capillary pipette inserted under the cover slip and operated by a rubber tube held in the mouth. The drop is in contact with the cover slip above and the slide below, and diffusion occurs at its margins. By this method one can observe under a low power of the microscope all the details of the reactions. Macroscopic observations can also be made in test tubes or other containers, but they do not permit the more delicate determinations.

Specifically in the case of the sea urchin, sperm suspensions exhibit three kinds of reaction to egg secretions of the same species, viz.: (1) *Activation:* the spermatozoa are stimulated to increased activity. (2) *Aggregation:* the spermatozoa are attracted to the drop of egg secretion and gather in or around it depending on its concentration. (3) *Agglutination:* the spermatozoa become stuck together in temporary clumps. It will be shown below that these reactions are due to three separable constituents of the egg secretions.

It will be convenient to describe first the entire group of reactions that go on when a drop of the egg water is injected into a 1 per cent suspension of the spermatozoa of *Arbacia* according to the method outlined above. At the time of injection, the sperm suspension is of a perfectly uniform milky consistency and the movements of the spermatozoa are rather slow. A very violent reaction begins immediately around the drop of egg water. In the first second or two the spermatozoa within the drop are aroused to intense activity and small agglutinations of spermatozoa form which secondarily fuse together with the greatest rapidity to form larger agglutination masses for a period of three to five seconds, after which no more fusion of masses takes place; while this has been going on in the interior of the drop a ring of agglutinated spermatozoa has formed at the margin, and a clear zone relatively devoid of spermatozoa arises external to it. The ring is at first continuous, but it ruptures in numerous places in two or three seconds, and each segment contracts quickly to a rounded agglutinated mass. In a period of time varying from a few seconds to a few minutes, depending on the concentration of the egg water, the agglutination disappears.

The drop of egg water, 2–4 mm. in diameter, may be regarded as equivalent, in a chemical sense, to a much magnified egg, and the reactions of the spermatozoa to it as similar to reactions to the actual egg, with the exception that the solid surface is lacking. The three kinds of effects of the specific egg water are manifest. The aggregation effect, evidenced by the ring and clear zone with reference to the introduced drop, is entirely similar to the aggregation of spermatozoa of *Nereis* with reference to CO_2 and dilute acids (see below). Each of these forms of reaction may be analyzed separately.

A. ACTIVATION

The spermatozoa of some animals, e.g., *Nereis* and *Arbacia*, become active in normal sea water immediately, and the specific egg water causes no noticeable acceleration in the rate of their movements under optimum conditions; but if they have become inactivated, by accumulation of CO_2 for instance, they may be aroused to instantaneous renewed activity by addition of very little specific egg water; the presence of an activating substance in the egg water of these forms is undoubted. The spermatozoa of some other forms, for instance *Asterias*, are not active in sea water, but they may be aroused to intense activity by the addition of specific egg water. Loeb (1915) points out that the spermatozoa of both sea urchins and starfish are immobile in N/2 NaCl solution in which they will continue to live for several days, but the addition of specific eggs will in each case cause immediate and often intense activity. The activation effect is, however, not very strongly specific, for the starfish eggs will activate sea-urchin spermatozoa, though the reverse effect is apparently not so pronounced (Loeb, 1915). G. H. A. Clowes and E. Bachman (1921*a* and 1921*b*) have shown that an activating effect similar to that of the egg water is produced by distillates of egg waters of the starfish, sea urchin, and sand dollar. They suggest therefore that the activating effect of the egg water is due to volatile autolytic substances; and they note that propyl, amyl, and cinnamyl alcohols have similar activating effects on spermatozoa. Thus the activating substances appear to be of a very different nature from the aggregating and agglutinating substances which are not volatile. These authors fixed no evidence of specificity in the distilled substances.

The relationship between activity of the spermatozoa and the presence of egg secretions may obviously be significant for fertilization, because it is improbable that immotile flagellate spermatozoa can fertilize eggs.

B. AGGREGATION AND CHEMOTAXIS

There has been much discussion concerning the rôle of chemotaxis in the fertilization of the egg. After Pfeffer's experiments on the chemotaxis of spermatozoids of ferns (1884) there was a tendency to invoke this principle in explanation of the meeting of the animal egg and spermatozoön. (Cf. E. B. Wilson, 1900, p. 196; and Verworn, 1895, p. 425.)

There has, however, been a strong reaction against this theory. Buller (1902), after a rather elaborate study of the behavior of spermatozoa of sea urchins in the presence both of eggs and egg secretions, came to the conclusion that the meeting of the spermatozoa with the outer surface of the jelly surrounding the egg is "a matter of chance" and not due to chemotaxis; "the spermatozoa are probably not chemotactically sensitive to any substances." The passage through the jelly is probably directed in some non-specific way. He was indeed unable to find any specific form of behavior that governed the spermatozoa in the presence of the ova, and hence attributed the meeting of the gametes to their vast numbers and the unguided motility of the spermatozoa.

Buller's conclusions were largely determined by the fact that spermatozoa did not enter specific egg water (or other substances tested) contained in capillary glass tubes immersed in sperm suspensions.[1] It is probable that other forms of behavior of the spermatozoa, viz., the strong thigmotactic response and the agglutination reaction to specific egg water (see below) prevent such entrance. If, however, the method described above be used, one always obtains a specific configuration of the spermatozoa around the introduced drop which entirely escaped Buller's observation. Part of this configuration appears to the writers to be due to a positive chemotaxis to a constituent of the specific egg water; the agglutination which appears simultaneously, however, complicates the situation, so that it is desirable to get rid of the agglutinating substance. This can be done by fixing the agglutinating substance of the egg water by addition of a sufficient quantity of species sperm which can then be centrifuged off; or by other methods (e.g., boiling, see Lillie, 1913*b*, p. 557, and 1914).

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[1] Dakin and Fordham (*Brit. Jour. of Exper. Biol.*, 1924) repeated Buller's experiments and reached contradictory results. They affirm that chemotaxis is demonstrated for spermatozoa of sea urchins.

If a drop of egg water of *Arbacia*, with agglutinating substance removed, be injected into a sperm suspension of the same species a ring of active spermatozoa forms around the drop separated by a clear zone almost devoid of spermatozoa from the general suspension. If the clear zone be examined carefully under the microscope, spermatozoa may be seen swimming directly across it from the general suspension to the drop of egg water for some minutes. The clear zone thus gives the range of some directive influence proceeding from the drop.

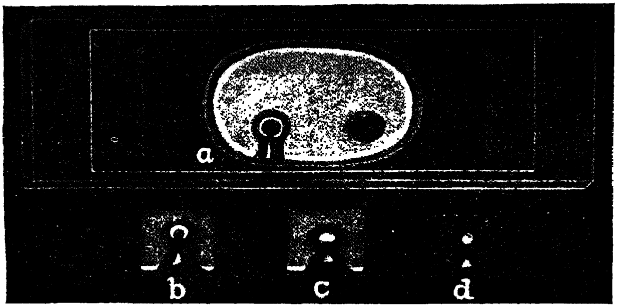

FIG. 14.—Reaction of a sperm suspension of *Nereis* to a drop of 1 per cent CO_2 sea water (natural size). The preparation (*a*) is mounted on a slide beneath a raised cover slip. *a* shows the form of the reaction after 15 seconds; *b*, 75 seconds; *c*, 105 seconds; *d*, 195 seconds. In *d* the general suspension has aggregated. The drop to the right in *a* is a control drop of sea water. Note in *a* that the spermatozoa also withdraw from the margin of the preparation, thus in the direction of increasing CO_2 tension.

The same reaction can be studied with greater advantage with spermatozoa of *Nereis* and a drop of 1 per cent CO_2 sea water. The configuration that immediately develops is shown in Figure 14. A ring of densely aggregated, very active, spermatozoa forms near the margin of the original drop, and a similar linear aggregation extends from this to the margin of the slide along the path in which the pipette was introduced and withdrawn. The clear space is 1.5 to 2 mm. in width. No such reaction takes place with reference to a drop of normal sea water. Greater dilutions down to 0.5 per cent of the CO_2 sea water will act positively in the case of fresh sperm suspensions, though then the ring lies within the margin of the drop. In the case of stronger CO_2 solutions the ring is farther from the center of the introduced drop, and tends to grow at the periphery as the CO_2 diffuses outward. In general, the aggregation tends to occur at a place in the CO_2 gradient near the point of paralysis of the spermatozoa.

The spermatozoa continue to move across the clear zone of such a preparation toward the central aggregation for some minutes. None move in the opposite direction. The behavior of the spermatozoa at the margin of the ring-shaped aggregation is similar to the ordinary thigmotactic reaction, i.e., they move very actively in circles of short diameter before coming to rest. The ordinary locomotion of spermatozoa consist of two components, forward and rotary, so that the path is spiral; thus at the margin of the aggregation the rotary motion predominates over the forward motion, and at this point in the CO_2 gradient the spermatozoa are practically imprisoned though active.

It is clear from this experiment that the spermatozoa of *Nereis* exhibit a positive chemotaxis in a CO_2 gradient of a certain range of concentration. To furnish such a gradient the concentration of CO_2 must exceed that in the sperm suspension, which is a function of its density and age; on the other hand, a limit is set to the differential necessary for the reaction by the fact that a concentration of about 1 per cent of the CO_2 sea water paralyzes the spermatozoa of *Nereis*.

The same configuration arises in response to egg water in echinids, as we have already seen; it must be similarly explained. If this is so, the width of the clear zone, about 2 mm., is a measure of the range of the chemotactic influence of the egg. Some observers describe some of the spermatozoa of a preparation as swimming by an egg within much shorter range without deviating toward it (cf. Buller, 1902; Morgan, Payne, and Browne, 1910); these observations can hardly be questioned. It is to be borne in mind, however, that the life of spermatozoa is very brief, and the period of their normal functioning presumably much less (cf. p. 476). Such observations cannot lessen the significance of the fact that spermatozoa do actually move up a gradient of egg water of increasing concentration.

How much significance such directive movements of spermatozoa may possess for the normal processes of fertilization is not clear. In artificial insemination the density of the sperm is controlled so that the meeting of the egg and sperm would be insured by entirely random movements of the spermatozoa; but this is probably not always so in nature, and the short directive range may be of significance. There is no reason for supposing that the chemotactic effect of egg water is very specific; however, data for determination of this point are lacking.

The effective surface of the egg for attaching spermatozoa is multiplied by the jelly in which the ova of so many forms are incased; and this acts as a trap to hold the spermatozoa both mechanically, and probably also by concentrating the agglutinating secretions of the egg upon them.

C. AGGLUTINATION

The agglutination of spermatozoa *to one another* by a constituent of the egg secretion as described in the beginning of this section (p. 452) is of course of no significance as such in fertilization. But it is certain that spermatozoa coming within effective range of the secretions diffusing from the egg undergo the physical and chemical changes involved in the agglutination phenomenon. These are, therefore, an invariable part of the fertilization reactions. However, the only method we have of studying the agglutinating substance is by the phenomenon of agglutination of the spermatozoa to one another. The fundamental fact is that fertilizable eggs of *Arbacia* in sea water secrete a substance (*fertilizin*) for which only the spermatozoa of the same species constitute an efficient indicator. Similarly, the only cells that produce the substance are the egg cells of the same species; it is not found in the blood or in extracts of other tissues (Lillie, 1914, p. 525).

The agglutination effect is a very definite and also characteristic reaction entirely different from mere aggregation of spermatozoa due to chemotaxis or other causes. In ordinary aggregations the spermatozoa are loosely associated, and slight agitation is sufficient to scatter them; in the agglutinated masses the spermatozoa are stuck together and are not separated by shaking. The agglutinating substance also produces its characteristic effect when shaken up and evenly distributed in a vial of sperm suspension, but aggregations cannot form in the absence of a gradient. The agglutination reaction is also spontaneously reversible unlike aggregation. After complete agglutination of a sample of spermatozoa and following reversal, the reaction cannot be repeated with fresh egg water owing to complete fixation of the agglutinable substance born by the spermatozoa.

The phenomenon of agglutination of sperm suspensions by egg water does not occur in all animals.[1] Its absence, however, should not be taken as evidence for the non-existence of a comparable secretion in such cases, for the adhesion of the spermatozoa to one another obviously depends on the magnitude of a certain physical change in the spermatozoa and on other conditions, which may not be realized in all forms; in such cases sperm suspensions cannot be used as indicators for the agglutinating substance; but to reason from this to absence of agglutinating substance would be illogical. Similarly, it is clear that the phenomenon of sperm agglutination as indicator must have its limitations at some point of dilution of the agglutinating substance; but as no other means is at present available for studying the agglutinating substance we are ignorant in comparative terms of what this limit may be. However, it is entirely possible that below this limit the spermatozoön may be sufficiently affected for purposes of fertilization.

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[1] The forms in which iso-agglutination of spermatozoa has been described are the annelid *Nereis* (Lillie, 1913), the sea urchins *Arabacia punctulata* (Lillie, 1913, 1914; Glaser, 1914; Just, 1922; and others), *Strongylocentrotus purpuratus* and *S. franciscanus* (Loeb, 1914; Lillie, 1921*a*; Sampson, 1922), the sand dollar *Echinarachnius parma* (Just, 1919), the starfish *Asterias forbesii* (Glaser, 1914; Woodward, 1918), and the chiton *Katharina tunicata* (Sampson, 1922). This list is not intended to be complete, and it should be emphasized that relatively few species have been examined

Loeb (1914) points out that sperm suspensions paralyzed by KCN do not agglutinate in specific egg water; thus motility of the spermatozoön appears to be necessary for the reaction; this is readily understood on the principle that energy of impact is necessary for adhesion. Loeb also states that the duration of the reaction is dependent to some extent on the alkalinity of the medium. "The more alkaline the latter, the more rapidly the cluster scatters. The presence of a salt with a bivalent metal, especially Ca, seems necessary for the cluster formation."

The agglutination reaction may be studied in a quantitative way. The reaction is reversible, as we have seen; with high concentration of the agglutinating substance it may be several minutes before the agglutinated masses break up and reversal is completed; with low concentrations, on the other hand, the agglutinated masses are smaller and their disintegration is correspondingly more rapid. It is, therefore, possible to determine a unit concentration of the agglutinating substance defined as the greatest dilution at which an unmistakable reaction is given. Such a reaction lasts only four or five seconds, and the agglutinated masses are too small to be seen with the unaided eye. Any given egg water may, therefore, be rated by the amount of dilution required for reduction to unit strength, as containing 10, or 100, or 6,400, etc., agglutinating units. The highest concentration obtained in *Arbacia* and in *Echinarachnius* is about 12,800 units.

The agglutinating substance is not contained in the blood (perivisceral fluid) or in extracts of any other tissues. We thus have a specific chemical relation between egg and spermatozoön that does not obtain between any other tissue and the spermatozoön. The reaction is tissue-specific as between egg and spermatozoön. The agglutinating substance is produced by mature eggs alone, and ceases to be produced by fertilized eggs in the case of echinids. In the case of *Nereis* it is produced by primary ovocytes, but these are fertilizable. Its period of production appears to coincide with the fertilizable period of the ovum. This point has been studied with great care in *Arbacia* and *Echinarachnius* (cf. F. R. Lillie, 1914, 1915; and Just, 1919*b*).

Loeb's suggestion (1914) that the agglutinating substance is merely the dissolved jelly that surrounds the egg may be disproved in two ways. First (specifically in *Arbacia*), it is possible to show that eggs deprived of their jelly either by treatment with dilute HCl which dissolves the jelly, or by shaking, which may remove the jelly mechanically, continue to produce the agglutinating substance as long as they remain fertilizable (Lillie, 1914, 1915). Just (1919*b*) has made the same determination for *Echinarachnius*. In the case of *Strongylocentrotus purpuratus*, however, as Loeb states, and one of us has confirmed (Lillie, 1921*a*), the production of the substance by eggs deprived of jelly is not sufficient to be detected by sperm suspensions, although the eggs remain fertilizable. This does not, however, justify the assumption that the substance is merely dissolved jelly, for it is not so in *Arbacia*, and the assumption is inconsistent with the second proof. This is that primary ovocytes of *Arbacia*, full grown but with germinal vesicle intact, although they possess the full complement of jelly do not produce a trace of agglutinating substance. The dissolved jelly of such eggs is always negative (cf. Lillie, 1915, pp. 26–27). The same is true of *Echinarachnius* (Just, 1919*b*). It follows, therefore, that the formation of the agglutinating substance does not begin before the time of maturation of the egg. It is presumably connected with the rupture of the germinal vesicle, for it is always present after maturation. Brachet (1922) shows that capacity for fertilization, or at least the first stages of the fertilization reaction, arises at this time in the sea urchin (cf. p. 478). The facts suggest, therefore, very forcibly a complete synchronism between origin of fertilization capacity and the first appearance of the agglutinating substance.

The agglutination is between the heads of the spermatozoa, which obviously become adhesive as a result of action of the egg water; the tails of the spermatozoa are apparently unaffected; the adhesive change apparently soon passes away, if we may rely on the evidence of reversal of agglutination. De Meyer (1911) observed that egg extracts of *Echinus*, which contain certainly other substances than the secretions of uninjured eggs, cause a strong swelling of the head of the spermatozoön, including the nucleus, which may increase the diameter as much as eight times.

Chemical and physical properties of the agglutinating substance.—The agglutinating substance is colorless; it does not pass through a Berkefeld filter, but passes readily through specially hardened filter paper; it is non-dialyzable; it is extremely heat-resistant, being destroyed only slowly at the boiling-point; it may be kept in sea water for months though it slowly disintegrates; it is obviously colloidal in its character, but Glaser (1914) has determined that it gives no clear response to usual protein tests although it contains carbon and nitrogen. Richards and Woodward (1915) point out that its agglutinating efficiency, like pepsin, varies with the square root of the concentration. The same authors also state that X-radiation affects solutions of the agglutinating substance in the same sense as ferments, accelerating in a short exposure (about two minutes), non-effective in a five-minute exposure, and inhibitive in a longer exposure.

A completely agglutinated sperm suspension in which reversal has occurred is not capable of reagglutination by addition of more of the agglutinating substance, and the substance disappears in an agglutinated suspension when not present in excess. The agglutination has, therefore, some of the usual characters of a chemical reaction. Glaser (1914) has also made the same determination. Schücking (1903) also holds to a union of the agglutinating substance of the egg with the agglutinable substance of the spermatozoön. Lillie (1914) has determined that 1 c.c. of a 3 per cent sperm suspension of *Arbacia* will fix all the agglutinating substance contained in 1 c.c. of egg water 64 units strong. Whether we are dealing here with a true chemical union or with a process of adsorption is not determined.

If sperm suspensions are allowed to stand for some hours they reach a condition where they will agglutinate only partially with strong solutions. If the binding power is then tested it is found that they fix as much of the agglutinating substance as when fresh. The fixing power of the sperm is thus entirely independent of its capacity for being agglutinated. The binding capacity undoubtedly depends on the presence of a special substance borne by the spermatozoa which we may call the agglutinable substance. It may be that in stale sperm suspensions this substance is cast off and lies free in the medium—a conclusion that would agree with our previous one (p. 477) that spermatozoa may loose a substance necessary for fertilization while they are still living and active.

Woodward (1918), who has confirmed most of the foregoing observations, believes that the sperm agglutinin may be precipitated from the egg secretion of *Arbacia* by $(NH_4)_2 SO_4$, as a flocculent whitish precipitate. By this method, she asserts, a solution may be obtained of greater agglutinating power than the original egg secretion, if the precipitate is washed free of ammonium sulphate and redissolved.

D. HETERO-AGGLUTINATION AND SPECIFICITY

The phenomenon that we have so far observed is the reversible adhesion of spermatozoa to one another by action of some constituent of the egg water of the same species. Agglutination in the sense of mere physical adhesion of the spermatozoa is by no means a specific action, for it may be produced by alkalies (cf. Lillie, 1913), by salts of trivalent metals (Gray, 1915), and by various other means including in some cases heterologous egg waters. Such agglutination is almost invariably toxic (but cf. Sampson, 1922) and non-reversible; it may, therefore, be distinguished sharply from the iso-agglutination which is non-toxic and reversible.

In order to estimate the rôle of iso-agglutination in fertilization it is important to know whether it possesses specificity comparable to the specificity of fertilization. In the first case studied as to specificity (Lillie, 1913, 1914), viz., the annelid *Nereis* and the echinid *Arbacia*, it was found that the egg water of *Nereis* did not cause any agglutination of *Arbacia* sperm, while *Arbacia* egg water causes a very firm irreversible agglutination of *Nereis* sperm; if the

Arbacia egg water be strong the spermatozoa of *Nereis* are rapidly killed. The blood serum of *Arbacia* produces the same effect on *Nereis* sperm, whereas it is an indifferent medium for the specific sperm. This suggests that the "hetero-agglutinin" and the "iso-agglutinin" of *Arbacia* egg water are separate substances.

This can in fact be demonstrated in more than one way. Thus in one experiment *Arbacia* egg water that originally acted on sperm suspensions both of *Arbacia* and *Nereis* was found to have lost all effect on *Nereis* sperm after seventeen days, whereas measurements showed that its iso-agglutinating power was not diminished. The non-specific substance was destroyed by the chemical changes in the egg water, but the specific substance remained. It is also possible to precipitate out all the *Nereis*-active substance with *Nereis* sperm and leave the full complement of iso-agglutinating substance (Lillie, 1913, 1914). The sperm of a teleost was also found capable of neutralizing the hetero-active substance, leaving the iso-agglutinating substance intact. The hetero-active substance would thus appear to be rather generally toxic to foreign sperm.

Just (1919*b*) has made a similar series of very detailed and careful experiments concerning the cross-agglutinating properties of *Arbacia* and *Echinarachnius* egg waters on one another. He finds that *Arbacia* egg water produces hetero-agglutination of *Echinarachnius* sperm, and that the reciprocal experiment is negative. The hetero-active substance in the *Arbacia* egg water may be precipitated out by *Echinarachnius* sperm leaving the iso-agglutinating substance intact. The hetero-agglutination in this case also is of a different character from the iso-agglutination, and apparently involves a toxic coagulation.

But the specificity of the iso-agglutinin is much more pronounced than these cases would indicate. Loeb (1914, p. 125) notes that the egg water of the sea urchin *Strongylocentrotus purpuratus* will agglutinate both its own sperm and that of *S. franciscanus*, but the latter not so strongly; however, on the other hand, *S. franciscanus* will agglutinate its own sperm but not that of *S. purpuratus*. Lillie (1921) studied the relations of sperm agglutination in these two species more fully. The egg water of either species may attain an iso-agglutinating strength of 640 units, but the egg water of *S. franciscanus* never has the slightest agglutinating action on the sperm of *S. purpuratus*, whatever its strength. On the other hand, the egg water of the *majority* of individuals of *purpuratus* may cause an apparent slight agglutination of the sperm of *franciscanus;* so that we have the appearance of a lack of agreement between the reciprocals in this case also. The egg water of certain *purpuratus* females has, however, not the slightest effect on *franciscanus* sperm, however concentrated it may be. This is not at all unusual. The hetero-agglutinating action is sporadic, while the iso-agglutinating action is constant. On the assumption that the iso-agglutinating action in *purpuratus* is caused by a single substance we cannot have this substance sometimes agglutinating the sperm of *francis-* and sometimes not, unless we assume that the variability is in the sperm of *franciscanus;* but this was shown by suitable experiments not to be the case. It must, therefore, follow that the egg waters of certain individuals of *purpuratus* contain a separate hetero-active substance.

Thus the phenomenon of agglutination of spermatozoa by a substance contained in egg secretions displays a high degree of specificity, higher, *apparently*, even than the specificity of cross-fertilization; for both reciprocals in the foregoing *Strongylocentrotus* crosses give a variable percentage of cross-fertilization. The appearance of greater specificity in cross-agglutination than in cross-fertilization may, however, be misleading owing to the difference in delicacy of the two indicators, spermatozoa and eggs. In the latter case each egg is a separate indicator of the success of fertilization; while in the former the indicator (sperm suspension) records nothing unless the change in the spermatozoa is sufficient to cause them to adhere. It is enough to have a clear demonstration that the specificity of sperm agglutination is at least comparable to the specificities in fertilization.

E. DISCUSSION

The fertilization reactions are considered under the following headings. It is obvious that in these reactions, which involve the meeting and fusion of the gametes, the effects of egg secretions on spermatozoa, which we have been considering, must play a part. The activation of spermatozoa by one constituent of egg secretion, the positive chemotaxis toward another constituent, and the agglutinable surface caused by a third constituent are forms of reaction suited to union. For reasons discussed beyond the specific agglutinating substance of egg water is identified with fertilizin.

3. *Relation of cortical reactions to the presence of fertilizin:*

We have already alluded to the first appearance of fertilizin in the course of ovogenesis, viz., at the time of rupture of the germinal vesicle in *Arbacia* and *Echinarachnius*, and the coincidence of its appearance with the origin of fertilization capacity (p. 488). During the fertilizable period it is produced in high concentration. After fertilization it ceases to be produced (Lillie, 1914; Just, 1919). In order to make the last determination it is necessary to remove the fertilizin-soaked jelly that surrounds the eggs; when this is done and the fertilizin present in the sea water with the eggs is washed off, no more is produced by the fertilized eggs. Similarly, we have shown for the same genera that eggs fully activated by butyric acid and which have become unfertilizable also produce no more fertilizin. There is thus a definite correlation between the secretion of fertilizin and fertilization capacity, which raises the presumption that the presence of this substance is essential for the fertilization reactions.

If this is so, artificial removal of fertilizin from the egg should result in the destruction of fertilization capacity. The first experiments to test this assumption were made by F. R. Lillie (1914) by repeated washings of the eggs in the attempt to remove the fertilizin. As a matter of fact, it was found that in *Arbacia*, even after removal of the jelly, the production of fertilizin was so persistent that it was impossible to reach a point in a series of over thirty washings lasting three days in which the eggs ceased to produce fertilizin. The amount produced, however, gradually decreases, and, along with this decrease, fertilization capacity is reduced. The objection that the eggs are otherwise injured during this time is certainly valid. Glaser has, however, found that the time for removing the egg exudates may be cut in half by use of running sea water, and that the same effect may be obtained in three or four hours by the use of charcoal (1921*a* and *b*). Such eggs do not fertilize. Thus a parallel is afforded between loss of fertilizing power and loss of fertilizin. Just (1919) showed that the eggs of *Echinarachnius* lose their power to secrete fertilizin much more rapidly than those of *Arbacia* in a series of washings, and that fertilization capacity falls off at a parallel rate. Four hours' washing with seven changes brings about an almost complete loss of fertilization capacity and fertilizin production. The quantity of production of this substance may be used as an index of fertilization capacity whether dealing with fresh eggs resistant to fertilization or with washed eggs in both *Arbacia* and *Echinarachnius*. Taken alone, we could only say that such a parallelism exists, and would hardly be justified in the assumption that fertilizin as such is a necessary link in the fertilization reactions. But taken in conjunction with the coincidence of time of appearance of fertilizin and fertilization capacity, and its disappearance after fertilization, the conclusion that fertilizin as such is necessary for fertilization seems highly probable.

Glaser (1921*a* and *b*) and Woodward (1918) have maintained that the addition of egg secretions to eggs that have almost lost fertilization capacity as a result of loss of secretion restores their fertilization capacity to a great extent. Glaser (1921*a*) notes that the complete removal of secretion from the eggs seems to have irreversible consequences. In other words, secretions, or fertilizin if this is principally concerned, cannot be entirely removed and restored at pleasure. We are, moreover, not convinced from the data given, that the inference of direct participation of added secretions in the fertilization reaction is justified in any case; it is entirely possible that added secretion acts by modification of the pH of the medium (see p. 506). The same criticism applies to Fuchs's (1915) somewhat similar conclusions.

4. *Sequence of events at the cortex of the egg in fertilization:*

The cortical reactions in fertilization determine the entire series of events. Fertilization cannot take place without these reactions (see p. 479), and the viability of the zygote is proportional to the intensity of the cortical reactions.

The first point is proved by the absence of results if the spermatozoön penetrates an immature egg (Lillie, Just, and others) which gives no cortical reaction or if it enters an endoplasmic sphere (Chambers, Just, Hyman). The second point has often been noted, the criterion being the completeness of formation of the fertilization membrane: those eggs in which the membrane is incompletely formed, or elevated less than normal, are weakly and do not develop far. As the initial and necessary stage of all the processes of fertilization, the cortical reactions deserve special attention.

The cortical changes themselves, although a small part in point of time of the entire series of events in fertilization, are complex. In the echinoderms, and presumably in other forms, they permit of subdivision in a time series as follows: (1) agglutination of the spermatozoön of the egg; (2) a latent period; (3) a sudden change in the properties of the egg or egg membrane that protects against polyspermy; (4) specific (presumably chemical) reactions (activation in the strict sense); (5) penetration of the spermatozoön (specifically in sea urchins); (6) membrane elevation.

In this section it is not proposed to analyze the series individually, but only to establish the validity of the periods distinguished in order to interpret the results of various experiments.

1. Agglutination is presumed from the changes that spermatozoa undergo in the presence of egg secretions, and from the fact that the jelly surrounding the egg contains these secretions in the most concentrated form.

2. In any lot of eggs inseminated at one time with sperm of sufficient concentration to insure a practically simultaneous agglutination of spermatozoa to all eggs, the time of visible response of individual eggs may vary by from one to several minutes in different lots depending on the physiological state of the eggs. This suggests a variable latent period following agglutination of the spermatozoa. The existence of such a latent period can be demonstrated by a reagent, like $CuCl_2$, which in a concentration of one part to 500,000 parts of sea water absolutely inhibits activation, but allows eggs already activated to develop (Lillie, 1921). If eggs are inseminated in sea water and transferred at intervals of a few seconds to such a $CuCl_2$ solution, we find that the percentage of activated eggs in the series of transfers does not suddenly rise to the maximum, but gradually attains the maximum over a period that may vary in different lots of eggs from thirty seconds to several minutes. The copper makes a clear distinction between these eggs that have not yet begun activation and those that have, and thus demonstrates conclusively the existence of a variable latent period, for all eggs had spermatozoa agglutinated to them at the same time.

3. In the case of the sea urchin and indeed in most forms, the initial protection against polyspermy is presumably the first and most rapid reaction of the egg. Eggs that are in their best condition remain monospermic even in the presence of very concentrated sperm suspensions when hundreds, or even thousands of spermatozoa, must reach the egg almost simultaneously. The selection of one of these typically excludes all the others, even when they are

very numerous, so that the change of the surface ("wave of negativity," Just) on which the rejection of supernumerary spermatozoa depends must be presumed to be very rapid. The variability of the latent period, however, renders any estimate of the rate of this change very doubtful. It must be considered probable that the rate of this change is less in some other forms, thus in the starfish (Chambers, 1923) and particularly in eggs that are naturally polyspermic (cf. p. 472). Moreover, the induction of experimental polyspermy by injuring the eggs is evidence that the rate of transmission of the wave of negativity is subject to considerable variation in any species.

4. We have divided the remainder of the period of the cortical events rather arbitrarily in the case of the sea urchin and sand dollar into three periods: activation, entrance of the spermatozoön, and membrane elevation. But, because in other forms the entrance of the spermatozoön may take place later (see p. 456) the essential distinction is between certain unseen physicochemical events (activation) and the visible cortical changes. It is important to notice that the activation processes proper begin at a point, the site of future entry of the spermatozoön, as inferred from the phenomena of membrane elevation, and spread over the surface of the egg from this point at a rate that can readily be followed by the eye in certain forms (cf. Just, 1919; see p. 455).

5. *Inhibition of fertilization:*

Study of inhibition of fertilization has proved valuable as a means of analyzing the initial phases of fertilization more particularly. We shall discuss, first, the inhibition by blood plasma and tissue exudates of the species; second, the inhibition by copper and salts of other heavy metals; and third, other means of inhibition.

A. BY BLOOD PLASMA AND TISSUE EXUDATES

Embryologists generally appreciate the fact that contamination of the eggs of some marine invertebrates with coelomic fluid or tissue exudates of the species reduces considerably the percentage of fertilization; it is, therefore, a common practice to wash the eggs once or several times before insemination. The inhibitory effect of coelomic plasma has been studied particularly by Lillie (1914) using the sea urchin, *Arbacia*. It may be shown in the case of this sea urchin by the following table:

Percentage of Coelomic Plasma in Sea Water	Percentage of Eggs Segmented
1	75
5	10
10	0.2
20	0.2
40	0.2
100	0
Control: same eggs in sea water	75

A series of dilutions of the filtered plasma was made as shown in the left-hand column; and identical inseminations of eggs of *Arbacia* were simultaneously made in each. An excess of sperm was used in each case. It is clear that the plasma in this case exerted a strong inhibiting effect on the process of fertilization. The inhibition operates on the initial stages because the eggs do not form membranes. Coelomic fluid of *Arbacia* does not always inhibit so strongly, and very wide individual variation with reference to this effect was found, which is possibly due to variation in the state of gonads. The same principle holds also for the starfish, according to Lillie. For example, in a series of dilutions of the coelomic fluid of the starfish, 10 per cent, 20 per cent, 40 per cent, 80 per cent, and 100 per cent, the percentages of segmentation following identical inseminations of the eggs of the same species were 62 per cent, 43 per cent, 50 per cent, 32 per cent, 11 per cent; 98 per cent, of the control eggs, in sea water, segmented.

Just (1923*b*) finds that eggs of *Echinarachnius* of high fertilization capacity fail to fertilize in the presence of coelomic fluid of the species. In essentials the results are comparable to those obtained by Lillie with eggs of the sea urchin and of the starfish.

If, however, one takes *Asterias* eggs which are resistant to fertilization in sea water, a condition often found, the addition of *Arbacia* coelomic fluid may cause a very high proportion to fertilize (cf. Lillie, 1919, p.174).

The action of the heterologous plasma in this case may be supposed to be due to a definite membrane effect enabling interaction between sperm and egg substances. This interpretation thus places the experiment in the same category with R. S. Lillie's (1911) ether experiments on *Asterias* eggs resistant to fertilization. This result excludes the interpretation that the inhibiting effect of species plasma may be due merely to colloid content, for this factor is common to both experiments, with the homologous as well as with the heterologous plasma. Moreover, the great variability of the effect of different samples of plasma—especially in the cases of *Arbacia* and of *Echinarachnius*—ranging all the way from 0 to 100 per cent inhibition also shows that we are not dealing with a general colloid effect.

How then does the inhibitor act—on the egg, or on the sperm, or by intervening in the reaction between the two? Experiments (Lillie, 1914) undertaken to answer this question showed that both eggs and sperm exposed to the plasma and washed free of it again were fertilizable. The plasma, therefore, merely interferes in some way with the reaction. Now since sperm of *Arbacia* agglutinate as readily in mixtures of *Arbacia* plasma and egg water as in egg water alone, Lillie concluded that it cannot be supposed that the plasma operates by preventing the adhesion of the spermatozoön to the egg, if this is brought about by agglutination. Indeed, Just (1922, 1923*b*) finds that sections of *Arbacia* and of *Echinarachnius* eggs inseminated (both straight and cross) in the presence of coelomic fluid, which fail to segment, reveal sperm attached to and within the cortex. Obviously, some other explanation must be sought.

There are two other possibilities: (1) the plasma might be supposed so to alter the egg cortex physically that activation is impossible despite the presence of the sperm; so far we have been unable to deal in an experimental way with this vague conception. Or (2), it might be supposed to inhibit the action of the activable substance (fertilizin) of the egg by deviation effect. If the latter be true, then it should be possible to prevent the inhibitory action of the plasma by first saturating it with the activable substance contained *ex hyp* in egg secretions. This is, indeed, true. If a sample of plasma rich in inhibitor be divided in two parts and one part be saturated with egg secretions (by adding a large quantity of eggs), it is found that this portion has entirely lost its inhibiting properties (Lillie, 1914; Just, 1919*b*).

Coelomic fluid has been found also to interfere with the initial response of the egg to agents of experimental parthenogenesis. Thus, *Nereis* eggs do not readily respond to treatment by elevation of temperature in the presence of blood or tissue exudates (Just, 1915*a*). Similarly, eggs of *Echinarachnius* in coelomic fluid give poor response to exposure to butyric acid in sea water as shown by the low percentage and the poor quality of membranes separated (Just, 1919*c*).

B. INHIBITION BY COPPER AND OTHER METALS

Salts of copper, and of certain other metals also, inhibit fertilization. Experiments with copper and mercury more especially have been of great service in separating the stages in the initial reactions between sperm and egg substance (see p. 493). Thus, Lillie (1921*c*) found that the presence of one part of copper chloride in 500,000 parts of sea water completely inhibits fertilization of the eggs of *Arbacia*. Previous exposure of the gametes to the action of copper chloride is not necessary for this result: if eggs and sperm are dropped simultaneously into the copper-containing sea water and thoroughly mixed at once, no reaction occurs, though the sperm are as active as in normal sea water. Usually the eggs give no indication of the beginning of the fertilization reaction; but if any egg does so, fertilization runs to completion in the copper solution and the egg segments. The inhibition is thus an "all-or-none" effect. At normal sperm concentration, i.e., one generally employed to insure maximum percentage of fertilization, no eggs fertilized; and inhibition is marked even at as low a concentration as one part copper chloride in 2,500,000 parts of sea water. If higher concentrations of sperm be employed, at the concentration of 1:500,000, small percentages of eggs, varying somewhat in different experiments, may fertilize. There is thus a certain virtue in an excess of sperm in the presence of the copper inhibitor (as is true of blood) which is somewhat difficult to understand[1] since only one spermatozoön penetrates usually. The inhibition by copper is reversible since eggs that have been exposed to copper, whether in the presence of sperm or not, may be fertilized after return to sea water, provided the exposure has not been long enough to injure their vitality too much.

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[1] It is possible that the slight beneficial effect of heavy insemination may be due to fixation of enough of the copper by the sperm proteins to cause an effective reduction of the copper acting on the eggs.

If eggs are fertilized in normal sea water and transferred to 1/500,000 copper chloride in sea water two or more minutes after insemination, they develop for several hours up to a late cleavage stage; the copper acts as a slow poison, however, so that eggs rarely reach the swimming stage. Eggs may, indeed, segment in 1/250,000 and 1/125,000 copper chloride in sea water if transferred five or more minutes after insemination in sea water; their rate of death is naturally increasingly rapid. Beyond the first five minutes after insemination the eggs do not appear to vary notably at successive stages in their sensitiveness to copper chloride within the range explored. There is thus a striking contrast between the action of copper in the first moments of fertilization and thereafter; copper has an effect that belongs to a separate order of sensitiveness on such early stages of fertilization as compared with the later.

The question then arises as to the time limits of the early inhibiting copper effect. These limits were fixed by a number of experiments in which the eggs were inseminated in sea water and then transferred at intervals to the copper solution (cf. p. 493); the percentage and quality of segmenting eggs were noted. These experiments revealed: (1) That eggs that have begun the fertilization reaction before transfer to the copper solution go through to cleavage, while those that have not begun it are instantaneously inhibited. Transfers made even four seconds after insemination include a considerable proportion of eggs which complete fertilization and segment; this proportion rises to normal with increase of time before transfer. (2) That eggs transferred from normal insemination to the copper chloride within the first two minutes do not give a complete membrane reaction. The perivitelline space, normally very wide, is extremely narrow often down to the point of invisibility. The viability of such eggs is bad even if returned to sea water again within a few minutes; the copper thus acts strongly also during the period of cortical discharge leading to membrane separation. If present at the time of insemination, it inhibits activation; and if its action begins within the first two minutes after insemination, it reduces the intensity of the cortical discharge proportionally to the time of its operation.

We may postulate the presence in the cortex of the egg of a copper-avid substance. On the hypothesis that this substance is fertilizin, the activable substance, we may say that copper checks the activation of fertilizin.

It may be noted further (1) that copper chloride in the concentrations employed in these experiments has no noticeable effect on the rate or duration of the sperm agglutination reaction. (2) Egg water protects against copper chloride inhibition much as it does against blood (p. 496); the same is true of gum arabic and of gelatin. (3) Copper inhibits activation by butyric acid. Mercuric chloride affects the initial stages but little as compared to copper, its deleterious effects increase as fertilization progresses.

Hoadley (1923), working with salts of eleven heavy metals, has obtained general results in accord with Lillie's copper and mercury experiments.

Numerous other substances may act as reversible inhibitors of fertilization, thus various salts, acids, anaesthetics. There are indications that many of these are similar to species plasma and copper in acting as inhibitors of the initial cortical reactions much more powerfully than as inhibitors of the following stages of fertilization. All agents so acting thus serve to differentiate the initial reactions physiologically from those that follow, and hence open the way to an analysis of the subject.

6. *Analysis of events following the initial reaction:*

After the agglutination of the spermatozoön to the egg and the following latent period (p. 493), the fertilization reactions are set in motion. The cortical changes, involving such morphological effects as the extrusion of jelly or the lifting of the membrane, etc., will be considered first; the chemical and physical changes that follow, e.g., in rate of oxygen consumption, in permeability, in viscosity, etc., will be considered separately.

A. ALTERED PHYSIOLOGICAL STATE OF THE EGG DURING THE VISIBLE CORTICAL CHANGES

The morphological changes in the cortex of such ova as those of *Ascaris*, echinids, *Nereis*, and *Platynereis* have already been noted (see p. 453). The altered physiological state of the egg at this time has been investigated chiefly by Just (1919*a* and 1922*c*). He finds that the egg of *Echinarachnius* is about twelve times more susceptible to exposure to tap water during membrane separation than the uninseminated egg, or the inseminated egg exposed before membrane separation begins: two minutes after insemination when the membrane is fully separated and equidistant from the egg at all points, the susceptibility falls suddenly to about one-eighth of what it was a few seconds previously. From this time the susceptibility falls with a minor and a major rise related to the mitotic process (Just, 1922*b*). Spaulding (1904) found in *Arbacia* eggs soon after insemination a lowered resistance to ether which is doubtless related to the cortical changes. Herlant (1920) reported similar results.

Just has been unable to discover a similar drop in resistance to tap water during the period of membrane separation in the egg of *Arbacia:* uninseminated eggs and eggs just lifting membranes show little differences in the rate at which they cytolyze. This is doubtless due in part to the size of the egg and to the rapidity with which the membrane separates. More recently, however, he has followed through to the swimming stage *Arbacia* eggs returned to sea water after exposure to tap water for varying lengths of time at short intervals after insemination. These experiments reveal that eggs exposed to tap water at the time of membrane separation give rise to characteristically abnormal blastulae and gastrulae; inseminated eggs exposed for the same time before and after membrane separation give normal larvae. There is here, therefore, evidence of a *differential susceptibility* which appears in clear-cut fashion during the later stages of development. Tap water exerts a decidedly deleterious effect during membrane elevation, and the egg is so profoundly altered that the normal processes of gastrulation are disturbed. These abnormal swimming forms are practically identical with certain of those obtained by Child with the use of various agents to modify the development of *Arbacia* eggs.

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The inseminated egg of *Nereis*, Just also finds, remains about five times more susceptible than the uninseminated egg to dilute sea water for about twenty-five minutes. This period almost exactly parallels the period of cortical changes marked by jelly extrusion, amoeboid activity (with changes in the shape of the egg and apparently a diminution in size), obliteration of the perivitelline space, and darkening of the plasma. With the resumption of the spherical shape and clearing of the plasma, the egg becomes exceedingly resistant to the action of dilute sea water.

These experimental findings on the ova of three forms, though they vary, all point to one conclusion, namely, that the period of cortical changes following insemination is one of profound physiological alteration.

Before the membrane lifts from the egg surface (echinid ova, for example) it is a living part of the cell; once it separates it is no longer a part of the metabolic machinery. Before insemination the vitelline membrane is the semi-permeable membrane; after it separates the hyaline layer takes its place. During the progressive dissolution of the colloids in the cortex, the egg is thus without adequate permeability regulation.

The escape of substances from ova at fertilization recorded by several workers has been cited as evidence of change in permeability at this time. The extrusion of jelly by *Nereis* eggs has already been mentioned. According to Reighard (1893), the egg of the wall-eyed pike excretes a substance from its cortex at the time of fertilization. Various workers, for example, Lyon and Shackell (1910), have cited escape of pigment from echinid ova at the time of fertilization as evidence of increased permeability, a conclusion that has been criticized by McClendon (1910).

Diminution in size of the egg (*Arbacia*) following fertilization due to loss of material has been reported by Glaser (1914), but no other worker on echinoderm ova has reported any such change in size. Okkelberg (1914), however, has determined that fertilization produces an average decrease of 13.48 per cent in the volume of the eggs of the brook lamprey.

The rate of oxygen consumption must likewise be affected during the period of cortical breakdown leading to membrane separation. Indeed, Shearer (1922) has reported a marked increase in oxygen consumption for the egg of *Echinus* immediately after insemination. He states (p. 214):

> There is a great initial inrush of oxygen into the egg and a corresponding output of CO_2 within the first minute after addition of the sperm to the egg. It is clear that the spermatozoön sets up an instantaneous oxidation process in the egg, which is perhaps unparalleled in the reactions of the animal cell for its sudden character.

B. PHYSIOLOGICAL CHANGES FOLLOWING THE CORTICAL REACTIONS

With the formation of the hyaline plasma layer, the egg, *Arbacia*, for example, may be said to be well started on its first cleavage cycle. Studies of the effects of fertilization on permeability, oxygen consumption, and viscosity have for the most part been made after the establishment of this layer; they therefore belong rather to the physiology of cell division than to the more specific events of fertilization.

McClendon (1910) demonstrated an increase in permeability by determining the electrical conductivity of *Toxopneustes* eggs before and after fertilization. Gray (1913) has made similar determinations. Lyon and Shackell (1910) have shown that eggs become more permeable to certain *intra vitam* dyes after fertilization. (Cf. Courier, 1923.)

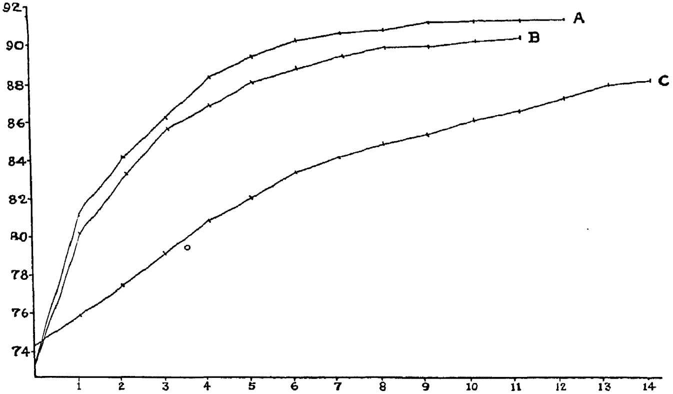

FIG. 15.—Imbibition of water by *Arbacia* eggs in diluted sea water (60 volumes tap water plus 40 volumes sea water). The curves are measurements of diameters at different intervals after placing in diluted sea water. Ordinates are diameters in micra, abscissae minutes after placing in the hypotonic medium. *A*, fertilized eggs; *B*, eggs with artificial membranes; *C*, unfertilized eggs (after R. S. Lillie).

R. S. Lillie (1916, 1918) has shown that ten to twenty minutes after fertilization *Arbacia* eggs take up water several times more rapidly than before fertilization. The volume of water entering the fertilized egg in three minutes was found to be $11 \times 10^5 \mu^3$ and the unfertilized egg, $3.6 \times 10^5 \mu^3$. Osmotic equilibrium is reached within about eight minutes in the case of the fertilized egg; in the case of the unfertilized egg the entrance of water is more gradual, and many eggs break down before osmotic equilibrium is reached. Figure 15 furnishes a comparison of the rate of entrance of water.

The rate of entrance of water is essentially constant during the period of exposure in the case of both fertilized and unfertilized eggs relative to the existing gradient of osmotic pressure, which demonstrates that the differences between the two sorts is due to a difference in the resistance of the membrane to the passage of water. The amount of water which enters or leaves the cell is finally the same in both fertilized and unfertilized eggs, thus again demonstrating a membrane effect. In the reverse experiment, of subjecting fertilized and unfertilized eggs to the action of hypertonic sea water, R. S. Lillie (1918) has shown that fertilized eggs lose water much more rapidly than unfertilized, as is to be expected.

The behavior of the fertilized eggs of *Echinarachnius* to hypotonic and hypertonic sea water is similar to that of *Arbacia;* but the egg of the starfish *Asterias* shows little difference before and after fertilization (R. S. Lillie, 1918). A comparable contrast with reference to oxygen consumption in the sea-urchin and starfish eggs respectively has also been found.

Warburg (1908-14) determined that the rate of consumption of oxygen by fertilized eggs of the sea urchin is six to seven times that of unfertilized eggs, and that the rate of oxygen consumption increases progressively for some time. Shearer's (1922) experiments have already been discussed. Loeb and Wasteneys (1912–13) found the increase in fertilized eggs of *Strongylocentrotus purpuratus* to be four or five to one as compared with unfertilized eggs. They did not, however, find any significant change in the starfish egg after fertilization. These authors also determined that membrane separation by artificial means causes a comparable increase in the rate of oxidation. The materials of comminuted unfertilized eggs of sea urchins (mechanical [Warburg, 1914], or by cytolysis [Loeb and Wasteneys, 1913]) also show an equal or greater increase in oxygen consumption as compared with an equal amount of intact unfertilized eggs. It would therefore appear that the material of unfertilized eggs is in a highly oxidizable condition. Comminuted material of fertilized eggs consumes no more oxygen than intact fertilized eggs.

The inference has been drawn from these oxidation studies that the same metabolic activities present before fertilization are accelerated, and this accelertion is the "cause" of fertilization. But the fact that in the starfish egg there is no appreciable increase in oxygen consumption, although we have the same phenomenon of initiation of development, sets this conclusion in a rather doubtful light. It is probable that in the case of the starfish egg there is an increase in the rate of oxygen consumption during maturation, thus before and independent of fertilization. Rate of oxygen consumption and fertilization are thus not of necessity causally connected.

Mathews (1906) has made a comparative study of the effects of KCN on fertilized eggs of the sea urchin and the starfish. He found in the case of the sea urchin a rhythm of alternate increase and decrease of susceptibility to KCN which accompanies the mitotic process. He could not determine in the starfish egg these sharp periods of susceptibility.

These data on permeability and oxygen consumption in the fertilized egg seem to be related to the processes of mitosis which are in progress at the time.

And indeed there is abundant evidence to show that rhythmical changes both physical and chemical accompany the cell-division process. These changes may be revealed by study of the susceptibility of the egg to hypotonic sea water (R. S. Lillie, Just, Herlant, 1920, etc.), to hypertonic sea water (A. R. Moore, 1915, Herlant) and to poisons (Lyon, 1902; Mathews, 1906; Spaulding, 1904; Baldwin, 1920; etc). Vlès (1921) has recently studied the changes in refractive index of the egg after fertilization.

Viscosity changes during mitosis have been studied by several workers using various methods. Among the most recent of this work on animal ova may be mentioned the studies of Heilbrunn, Chambers (1917), and Seifriz (1920). Heilbrunn (1915, 1920*a*, 1921) particularly has measured the magnitude of viscosity changes which he relates causally to the mitotic process.

7. *Superposition of fertilization on parthenogenesis:*

Loeb (1913*a*) reported that eggs of *Strongylocentrotus purpuratus* from which membranes had been induced to separate by butyric acid could be fertilized by sperm if the membranes were torn by shaking; they differed, therefore, in this respect from eggs in which membrane formation had been induced by fertilization. He also determined that eggs of the same form when treated with hypertonic sea water alone might begin development, but come to a standstill in the two-, four-, eight-, or sixteen-celled stage, and that such eggs were fertilizable in the sense that insemination may cause the formation of a separate membrane around each blastomere; they then resume development in a perfectly normal way, according to Loeb, and become normal larvae.

These results can be readily understood in the quantitative sense of partial activation. If the cortical reactions are incomplete in any experiment the possibility of superimposing fertilization on such incomplete reactions might remain. C. R. Moore (1916) has shown that such a quantitative relation actually obtains. To understand the experiments and results it is essential to examine the character of artificial activation by means of butyric acid, which was the agent used in both Loeb's and Moore's experiments. The strength of the acid and time of exposure are variable factors. Fifty c.c. sea water plus 2.8 c.c. N/10 butyric acid was used throughout the experiments. There is no visible change in the eggs while in the butyric acid; but when transferred to sea water, after proper exposure, the eggs separate membranes as they do after insemination, and they develop normally if subsequently treated with hypertonic sea water. By varying the time of exposure to the butyric acid we get graded degrees of activation as expressed in membranes separated by the eggs and in their capacity for development; this proceeds up to the optimum. If the exposure to butyric acid be prolonged beyond the optimum the capacity for development gradually falls off to zero.

It is clear that butyric acid produces a condition of pre-activation in which the egg activates when returned to its normal environment; but if the treatment be too short the pre-activation is insufficient; if it be too long some other condition arises that inhibits normal activities.

Insemination of such eggs after their return to sea water is found to be successful in inverse proportion to the degree of activitation induced in the sea water up to the optimum point, at which superimposed insemination has no effect. Beyond this the curve of superimposed fertilization shows a second rise and fall to zero. At the optimum point for artificial activation, at which all eggs form membranes and are capable of development after treatment by hypertonic sea water, the sperm has absolutely no fertilizing effect on the eggs, whatever its concentration, even if the membranes formed by butyric acid be entirely destroyed. Such eggs are, therefore, comparable to normally fertilized eggs in respect to their unfertilizable condition.

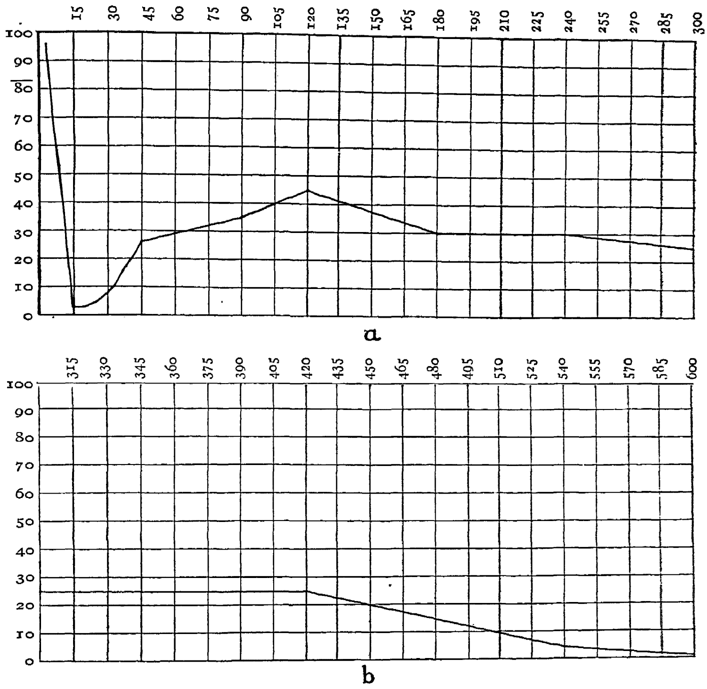

FIG. 16.—Curve of fertilization superimposed upon butyric acid treatment in *Arbacia*. Curve *b* continues curve *a*. The ordinates represent percentages of fertilization as measured by cleavage, and the abscissae length of previous exposure to butyric acid in seconds (after C. R. Moore).

Thus in the case of *Arbacia* on which these experiments were performed, there is no possibility of superimposing fertilization upon parthenogenesis after optimum exposure to the activating agent. But if the exposure to the activating agent be too short, or too long, some degree of capacity for fertilization exists which is expressed in Figure 16. Just (1919*c*) has examined this question of the superposition of fertilization on the butyric acid activation of *Echinarachnius* eggs, and has obtained results confirming Moore's. Lillie (1921*b*) has repeated these experiments on the same species of California urchin that Loeb used. He concludes that the membrane reaction after treatment with butyric acid is the same as after insemination, as shown by the similarity of the membranes and their rate of formation in the two cases. The result of rendering the egg insusceptible to spermatozoa shows a possible slight difference only; and this is obviously explained on the assumption that there is a variable tendency toward incompleteness of reaction after treatment with butyric acid which in rare cases leaves some fertilizin free for sperm action.

Moore (1917) has also examined Loeb's statement concerning the fertilization capacity of eggs previously treated with hypertonic sea water; he finds that here, too, the insemination of eggs that have received optimum treatment for experimental parthenogenesis does not increase the percentage of development. In the main, Just (1922*d*, 1922*e*) has confirmed these conclusions. According to this author, if *Arbacia* eggs be treated with strong hypertonic sea water, they separate membranes while in the solution (cf. Heilbrunn, 1924)—a fact first noted by Loeb, confirmed by Moore—and insemination does not increase the percentage of development. If, however, they are subjected to weaker hypertonic solutions and at any time after return to sea water, up to the terminal stages of mitosis, are inseminated, the percentage of development is increased. This finding is readily explained on the assumption of incomplete cortical reaction, since after the treatment the eggs produce fertilizin and since on insemination they separate normal membranes.

We may conclude from this rapid review of experimental evidence that the superposition of fertilization on experimental parthenogenesis is not possible if the experimental agent induces complete activation. Complete artificial activation is thus in no wise different from the initial activation by sperm. Once fertilized, or artificially activated, the egg cannot be fertilized. But it has been held that the experimental activation can be reversed.

8. *Reversibility of experimental parthenogenesis:*

Loeb (1913*b* and 1915*a*) found that alkalies induce development of eggs of *Arbacia* in a manner somewhat similar to that of butyric acid; but if the eggs after treatment for the proper length of time are put into a solution which prevents their development (sea water with chloral hydrate or NaCN) when taken out they behave as though nothing had happened to them. He considers this a demonstration that artificial activation can be reversed. Now it is notable that in such treatment no visible change occurs in the alkali, but only after transfer to sea water. It would seem, then, to be a reasonable interpretation that such changes are prevented by the chloral or NaCN. What is reversed, therefore, in this case is at most a condition which permits cortical changes in sea water. If eggs are placed from the alkali in sea water, even if only for a few minutes, before immersion in the chloral or NaCN sea water they will not reverse. It is apparent that such results can be as readily understood in the quantitative sense of partial or arrested activation as in terms of reversal.

9. *External conditions of fertilization:*

The fertilization reaction is dependent, not only on the internal conditions of the gametes, but also on the nature of the medium in which insemination occurs. Fertilization always occurs in an aqueous medium containing a balanced solution of salts, of which NaCL, $MgCl_2$, KCl, and $CaCl_2$ are the chief. In such a medium fertilization depends, within the usual range of temperature, on reaction of the medium (acidity or alkalinity) and the balance and concentration of the salts. Sea water is a medium of this kind in which the variable factors can be readily controlled. Most studies have, therefore, been made on marine animals; but there is evidence that the same principles apply to other animals.

A. THE INFLUENCE OF H-ION CONCENTRATION ON THE FERTILIZATION REACTION

Certain observers have noted that increase of acidity of the medium blocks fertilization, but that increase of alkalinity on the whole favors it within certain limits (cf. Lillie, 1919, pp. 170–71). In acid sea water there is a distinct block in the mechanism of fertilization, but the interference that appears in alkaline sea water seems to operate not on the fertilization reaction directly, but by impairment of subsequent processes. Very recently H. W. Smith and G. H. A. Clowes (1924, cf. also Clowes and Smith, 1923) have made a very complete investigation of the pH range of fertilization, and of the range of viability of gametes and zygotes of *Arbacia*, *Asterias*, and *Chaetopterus*. Believing that CO_2 exerts a profoundly specific effect apart from the H ions produced by its dissociation, these investigators have worked with CO_2-free sea-water solutions of different H-ion concentrations.

The results for *Arbacia* are shown in the accompanying curves (Fig. 17) kindly placed at our disposal by Dr. H. W. Smith who also adds the following comments:

> It was found that a block to fertilization appeared very abruptly at a definite H-ion concentration, which was constant for each species studied but which differed for the different species. Thus a block in the case of *Arbacia* appears at pH 6.8; in the case of *Asterias* at 7.0, and of *Chaetopterus* at 7.1. This block is perfectly reversible since the eggs which fail to fertilize on the acid side of the block readily fertilize when returned to sea water or to a H-ion concentration less than that limiting

fertilization. The sperm apparently do not react with the eggs in the acid solutions in any way, though they retain their motility and continue to congregate around the egg.

In sea-water solutions of greater alkalinity than pH 10.2, $Mg(OH)_2$ is precipitated, and therefore pH 10.2 is the limit on the alkaline side to which sea water can be raised without materially altering its chemical composition. From pH 8.0 to pH 9.8 to 10.0, *Arbacia* and *Asterias* eggs fertilize readily. At pH 10.0 in the case of *Arbacia*, and pH 10.2 in the case of *Asterias*, membrane elevation no longer occurs so long as the eggs are left in the alkaline solution. If returned to sea water within five to ten minutes, the majority of the eggs lift normal membranes and develop normally. From several considerations, it is believed that there is no alkaline block to fertilization; or, if such a block exists, it partakes of the nature of an impairment of the fertilization process, rather than a complete prevention of this process as in the case of the acid block.

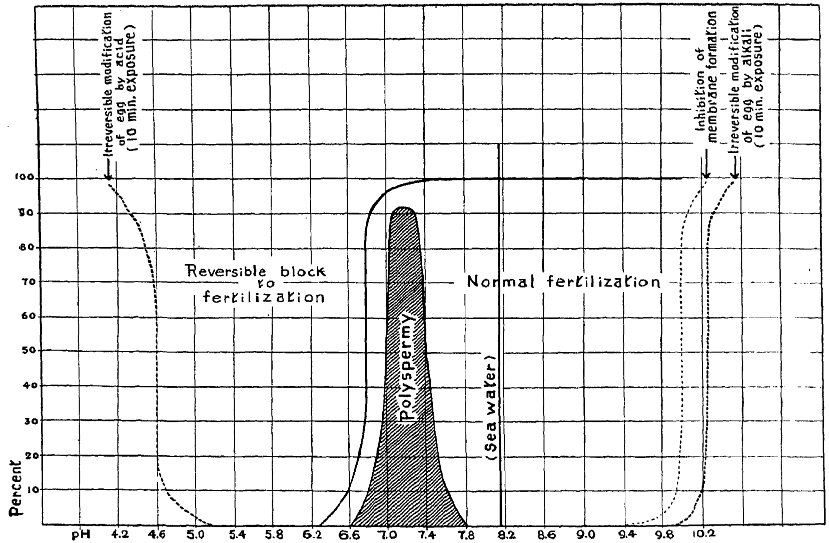

FIG. 17.—Diagram to show the influence of H-ion concentration on the fertilization of *Arbacia* eggs with *Arbacia* sperm. The heavy line shows the limit of fertilization, the shaded area the region of polyspermy, and the dotted lines the acid and alkaline limits in which the eggs are irreversibly modified by the solution alone. The heavy ordinate indicates the pH of sea water (8.15). (After Smith and Clowes.)

The limits of acidity and alkalinity to which these eggs may be exposed for ten minutes without irreversible injury, as shown by the retention of the capacity to fertilize when returned to sea water, are also defined in the diagram. The acid limit for a ten-minute exposure in both species is about pH 4.4; the alkaline limit is 10.2.

All our results indicate that the acid block to fertilization is in no sense an artificially imposed obstruction, but represents some physiological alteration of the mechanism of fertilization. This mechanism is apparently in direct equilibrium with the H×OH ion equilibrium of the surrounding medium. On the alkaline side of the block, all the eggs are fertilizable; on the acid side of the block none of the eggs are fertilizable; in the neighborhood of the block, eggs either fertilize and develop normally, or do not fertilize at all, showing that the fertilization process proceeds according to an all-or-none law.

In *Arbacia* there is a greatly increased incidence of polyspermy at pH 7.2. Though the incidence of polyspermy at all H-ion concentrations increases with increasing age or staleing of the egg, nevertheless within a very narrow range, centering at pH 7.2, nearly all the eggs will be polyspermic even when they are fresh and when the incidence of polyspermy is practically zero at H-ion concentrations greater or less than this.

In the case of the starfish and *Chaetopterus* the general pH relations are the same, but the absolute values are slightly different. Thus the point for fertilization of 50 per cent of the eggs which lies at 6.8 in *Arbacia*, is at 7.0 in the starfish and at 7.1 in *Chaetopterus*. Other values are also slightly shifted in the same sense.

Clowes and Smith (1923) also find that sea-urchin eggs that are not fertilized in the pH solutions between 7.0 and 6.4 are subsequently fertilized by adherent sperm carried over on return to sea water. Starfish eggs that just fail of normal fertilization in the pH solutions are so modified that they do not subsequently fertilize on return to sea water even with fresh sperm. Clowes and Smith conclude that in the sea urchin the entry of sperm appears to follow an all-or-none law. In the starfish a partial entry may be effected with a consequent permanent injury to the egg. It seems to us that this conclusion would be warranted only if experiments had been made on the effect of exposing the eggs to the solutions alone; the failure of fertilization on return to sea water may perhaps be due to the modification of the egg, within the given range, by the pH alone.

Increase of alkali has been noted by various authors to favor the reaction between the egg and spermatozoön, possibly because it tends to make the plasma membrane more permeable and thus to permit a closer relation between the activable substance of the egg and the spermatozoön. The use of a hyper-alkaline medium to facilitate fertilization was first made by Loeb (1903, 1904) in a successful attempt to produce heterogeneous hybridization, and has since been widely extended for the same purpose. It should be noted that the reaction is favored only in the actual presence of the alkali; previous treatment of eggs alone, of sperm alone, or both, before insemination in normal sea water does not increase the percentage of fertilization. We are probably dealing with a rapidly reversible modification of the surface of one or both kinds of gametes.

It may not be amiss in this connection to refer to the observation of R. S. Lillie that the concentration of butyric acid in sea water which induces the starfish egg while undergoing maturation to develop will inhibit the initiation of maturation in the ovocyte. Fauré-Fremiet (1921) finds that neutralization or slight acidification of the sea water inhibits maturation in the eggs of *Sabellaria* (*S. alveolata* and *S. spinulosa*). Miss Brailey (1923) finds that CO_2 inhibits maturation of the starfish egg. According to Hörstadius (1923), alkalinity is favorable to the maturation of the eggs of the Serpulid, *Pomatocerus triqueter*. Thus in one experiment the percentages of maturation at pH 7, 8, and 9 were 0.6, 25, and 31 respectively; in another the percentages at pH 8.3, 9.1, and 9.6 were 68.5, 85.6, and 86.6 respectively. The pH of the coelomic fluid was found to lie between 6.8 and 7.2.

B. BALANCE OF SALTS

With reference to the question of the balance of salts, the most important experiments are those of Loeb (1914, 1915*b*), who found that, for the fertilization of eggs of the sea urchin, the presence of Ca- and OH-ions is very important. Eggs and sperm washed in neutral N/2 NaCl will not fertilize in this salt alone, nor in combinations of two or more of NaCl, $MgCL_2$, and KCl in the proportions and concentrations in which these salts exist in sea water, though the sperm may be very active and fill the jelly of the eggs. But the addition of $CaCl_2$ to NaCl, or to NaCl and $MgCl_2$, or to NaCl $MgCl_2$ and KCl in the sea-water proportions will induce normal fertilization; this will happen even more promptly and certainly if a little NaOH is added at the same time. Loeb states (1914) that calcium possesses an almost specific action for fertilization of the sea-urchin egg, and it is important to note that it increases sperm agglutination also, according to the same author.

The spermatozoön, as we have pointed out previously, remains external to the egg in the case of *Nereis* and of *Platynereis* for some minutes after it has become stuck to the egg surface at the time of insemination. These ova, particularly that of *Nereis*, are therefore ideal for experimental study of the conditions determining the entrance of the spermatozoön into the egg. Just, in some hitherto unpublished experiments made during the summers of 1915 and 1917, has investigated the ability of the spermatozoön to penetrate *Nereis* eggs in various salt solutions isotonic with the sea water at Woods Hole. The solutions employed were: 0.52 M NaCl, 0.52 M KCl, 0.29 M $MgCl_2$, and 0.29 M $CaCl_2$. Eggs inseminated in the $MgCl_2$ and $CaCl_2$ solutions undergo no cleavage and produce no swimmers. Four hours after insemination most of these eggs are still in the germinal vesicle stage with sperm attached to the membrane. In time the eggs disintegrate. Eggs inseminated in the NaCl and KCl solution show some cleavage, about 1 per cent, and four hours after insemination many are disintegrating. About 1 per cent abnormal swimmers are produced. Sea-water inseminations all give 100 per cent cleavage and swimmers.

Eggs inseminated in NaCl and KCl and transferred to sea water at intervals up to eighty minutes after insemination develop to the swimming stage, the percentage depending upon the length of exposure. The time to first cleavage is the same as that of eggs inseminated in sea water at the time eggs are removed from the solutions. The swimmers show abnormalities.

The eggs inseminated in $MgCl_2$ and $CaCl_2$ solutions and transferred to sea water at intervals up to eighty minutes show a higher percentage of cleavage and swimmers. The time to first cleavage is the same as that of eggs inseminated in sea water at the time of transfer of eggs from the solution.

10. *The problem of specificity in fertilization:*

Study of the capacity of ova to respond to insemination with foreign sperm gives a measure of specificity in fertilization; any attempt at analysis of fertilization must account for this phenomenon. Fertilization is conditioned, not only by the physiological condition of the gametes and the composition of the external medium, but also by special properties of the egg and sperm belonging to them by virtue of taxonomic specificity. It was shown in our previous considerations that the fertilization reaction depends primarily upon cortical conditions and reactions both inherent and conditioned by the medium. The following data show that specificity likewise is primarily a function of the cortex.

Fertilization is tissue-specific and species-specific. Tissue-specificity is evident in the failure of the spermatozoön to react with other kinds of cells than egg cells. In the fertilization of *Ascaris*, for example, though innumerable spermatozoa may be found throughout the length of the oviduct among the epithelial cells they never enter these. It is noteworthy in this connection that sperm agglutinin is never produced by other than egg cells. Thus, Lillie (1913, 1914) was unable to find in the blood or in any of the tissues of either *Arbacia* or *Nereis* any sperm-agglutinating substance; the eggs of these forms, however, when ripe for fertilization yield abundant agglutinin. It would therefore seem that one factor at least in tissue-specificity in fertilization is the chemical organization of the gametes.

Relatively little work, however, has been done on tissue-specificity. The case is otherwise with species-specificity, although here the work has been largely concerned with other aspects of the fertilization problem than activation. Fertilization is species-specific in a restricted sense only, since to a certain extent eggs may react to foreign sperm of closer or more distant taxonomic relationship. The measure of species-specificity is the relative capacity of the egg to respond to foreign and to species sperm. It is from this point of view that we shall consider the work on hybrid fertilization.

A. HYBRID FERTILIZATION

The possibilities of hybrid fertilization have been investigated chiefly among echinoderms, teleosts, and Amphibia. In certain other groups—as insects, birds, and mammals—mating behavior constitutes a serious obstacle to investigation. We may consider the data on cross-fertilization in the order: echinoderms, teleosts, and Amphibia.

a) ECHINODERMS

Interspecific crosses among the echinoderms have been made in the genus *Echinus* and *Strongylocentrotus*.

Shearer, De Morgan, and Fuchs (1913) crossed three species of *Echinus: esculentus*, *acutus*, and *miliaris*, in all possible combinations. The fertilization succeeded in all six combinations without any artificial aid by either increasing the usual concentration of the sperm or changing the chemical constitution of the sea water. Larvae were readily raised from all crosses, but only the crosses *E. esculentus* ♀ × *E. acutus* ♂ and *E. miliaris* ♀ × *E. acutus* ♂ gave normal sea urchins.

The cytology of these crosses was studied by Doncaster and Gray (1913) and by Gray (1913). The behavior of the germ nuclei was not always normal; thus, in the cross *E. esculentus* ♀ × *E. acutus* ♂, the male nucleus swells before conjugation, whereas this is not the case in straight *acutus* fertilization. Some elimination of chromosomes from the first cleavage spindle occurred in certain of the crosses. A curious fact was that this might occur in one reciprocal of a cross but not in the other. Thus in the cross *esculentus* ♀ × *acutus* ♂ the cytological events are perfectly normal; but in *acutus* ♀ × *esculentus* ♂ there was an invariable elimination of some chromosomes.

Lillie (1921*a*) has studied the measure of specificity in fertilization between two associated species of the sea-urchin genus *Strongylocentrotus*. In the case of both species—*franciscanus* and *purpuratus*—a higher concentration of sperm is required for any degree of cross-fertilization than for practically perfect straight-fertilization. Eggs of each species straight-fertilize at a sperm concentration which has no effect in cross-fertilization. This difference may be enormous. Thus, if we take the lowest sperm concentration in which any cross-fertilization occurs, we can use a mathematical expression of the relative "ease" of straight- and cross-fertilization of eggs from a given female. With this form of expression we may find cross-fertilization of *franciscanus* eggs 20 to 500 times more difficult than straight-fertilization; cross-fertilization of *purpuratus* egg 750 times more difficult than straight-fertilization. Increase of the temperature or of the alkalinity of the sea water did not overcome this strong specificity.

In addition to these interspecific crosses in *Echinus* and *Strongylocentrotus*, wider crosses within the order have been frequently made. Vernon (1900) alone attempted forty-nine out of a possible fifty-six cross-fertilizations between eight species belonging to seven genera of sea urchins; eleven of these gave no sign of cross-fertilization; of the remainder, nine gave only segmentation stages or blastulae or gastrulae, and twenty-nine lived to the stage of eight-day plutei.

In Vernon's cross-fertilizations a high sperm concentration seems generally to have been employed. The percentage of eggs fertilized was nevertheless small as compared with species fertilization; in many cases exceedingly small. In several instances the eggs were staled for several hours, even up to twenty-four, before fertilization with a resulting increase in the percentage of eggs fertilized; but in the case of *Sphaerechinus* ♀ × *Strongylocentrotus* ♂ this treatment was not successful. It is important to note that specificity always appeared with reference to the relative ease of fertilization with the specific and foreign sperm.

Tennent (1910) made eleven crosses within the order in which the reciprocals belonged to different genera, families, or suborders; other observers have made similar crosses within the order. These studies have been made from the point of view of heredity or chromosome behavior and, unfortunately for our purpose, fertilization problems have been referred to only incidentally as a general rule. They do show, however, that the chances of success of a cross cannot be postulated wholly on the systematic position of the species. Thus, Tennent reports that the cross between *Moira* ♀ and *Toxopneustes* ♂ belonging to different suborders takes place very readily and the larvae develop well. The reciprocal cross can also be made, but succeeds best if the *Toxopneustes* eggs are allowed to stand in sea water five hours before being fertilized. *Hipponoë* ♀ × *Cidaris* ♂ gives poor results; no fertilization membrane is formed, segmentation is irregular, larvae abnormal. Tennent also made the crosses *Cidaris* ♀ × *Hipponoë* ♂ and *Cidaris* ♀ × *Toxopneustes* ♂. The eggs may be fertilized at once after removal from the ovary without artificial aid. However, as a check against the possibility of error due to chance fertilization with *Cidaris* sperm, the eggs were kept for two hours before insemination.

Thus, reciprocal crosses are not always similar with reference to fertilization. A notable example is that noted by Fischel (1906): the cross *Strongylocentrotus* × *Arbacia* succeeds when *Arbacia* is the male, but never when *Strongylocentrotus* is the male. Practically the same relations hold for the cross *Echinarachnius* × *Arbacia* (Just, 1919*b*). Gemmill found the same to be true in the cross, *Cribrella* × *Asterias*. With reference to the *Strongylocentrotus* × *Arbacia* cross, Fischel states that the Hertwigs obtained the exact opposite of his result in another locality.

A difficulty arises in any attempt at analyzing the data on echinoderm cross-fertilization in that some authors state only their successful cross-fertilizations, and the facts with respect to the reciprocals are not stated. Baltzer (1910) has, however, paid particular attention to this question, and his results may be tabulated as follows:

	Echinus ♀	*Strongylocentrotus* ♀	*Sphaerechinus* ♀	*Arbacia* ♀
Echinus ♂		No elimination (1)	No elimination (2)	8–15 chromosomes eliminated first cleavage (4)
Strongylocentrotus ♂	No elimination (1)		No elimination (3)	8–15 chromosomes eliminated first cleavage (5)
Sphaerechinus ♂	16 chromosomes eliminated first cleavage (2)	16 chromosomes eliminated first cleavage (3)		16 chromosomes eliminated first cleavage (6)
Arbacia ♂	Chromatin eliminated blastula (4)	Chromatin eliminated blastula (5)	No elimination (5)	

NOTE.—The reciprocal crosses bear the same number.

Only three of these cross-fertilizations were successful in normal sea water, then only occasionally, and exact data are not given, viz., *Sphaerechinus* ♀ × *Strongylocentrotus* ♂; *Strongylocentrotus* ♀ × *Arbacia* ♂; *Echinus* ♀ × *Sphaerechinus* ♂. The fertilizations recorded were, therefore, made in hyperalkaline sea water according to Loeb's (1903, 1904) method. Very great variability in the capacity for hybridization even by this method was encountered as by other authors. Very little can be learned, therefore, concerning the kind and degree of the natural specificity of the fertilization reactions in these cases, except what has been stated, namely, that in nine out of twelve cross-fertilizations hyperalkalinity of the sea-water is necessary to permit penetration of the sperm. But, after the cortical specificity is broken down by the hyperalkaline medium, fertilization proceeds normally up to the metaphase of the first cleavage, when elimination of paternal chromatin occurs regularly in certain cases. In other cases this elimination is postponed until the blastula stage, and in yet others it does not occur at all. But there is only one case in which the reciprocals behave alike in this respect, viz., in the *Echinus* × *Strongylocentrotus* crosses among the six pairs of reciprocals.

Interclass crosses have also been made in echinoderms. Thus Loeb (1903, 1904) found that the eggs of *Strongylocentrotus purpuratus* can be fertilized by the sperm of *Asterias* in the presence of excess alkali; sperm of a brittle star was equally effective. About 50 per cent of the eggs formed membranes and segmented. Loeb could not succeed in fertilizing the eggs of *Arbacia* with the sperm of *Asterics* with this method. Starfish sperm affect the sea-urchin egg (*Strongylocentrotus*) only in the presence of the alkali; eggs or sperm previously treated with alkali will not react when brought together in pure sea water. The effect may be on the spermatozoön or on the egg or on both; but it is obvious that some surface reaction of the egg and spermatozoön is favored by the alkali, because when the sperm once gains entrance to the egg it calls forth the further necessary reactions within it.

Loeb (1903, 1904) also found that if the sea-urchin eggs are deprived of their jelly by the action of HCl they cannot be fertilized by starfish spermatozoa with the same use of hyperalkaline sea water that readily brings about fertilization in the presence of the jelly, although they are readily fertilized with their own sperm. But if an excess of calcium is added to the hyperalkaline sea water, the heterogeneous fertilization succeeds in the absence of the jelly, and in this case practically all of the eggs that form membranes segment. In the presence of the jelly many eggs form membranes but fail to segment owing to the failure of the spermatozoön to penetrate. Loeb suggests that the latter phenomenon may, therefore, be due to agglutination of the starfish sperm to the jelly.

Godlewski (1906) attempted to fertilize sea-urchin eggs with sperm of starfish, holothurians, and brittle stars by Loeb's method without success. However, he succeeded in fertilizing eggs of the same genera of sea urchins with the sperm of *Antedon rosacea* (crinoid) by the same method. The fertilizations succeeded best in the hyperalkaline sea water with a high concentration of sperm; but some eggs fertilized when first exposed and then washed in normal sea water, a fact that shows the main effect of the alkali to be on the egg. A few eggs might develop to normal plutei, thus exhibiting a purely maternal inheritance, in spite of the fact that the sperm nucleus fused with the egg nucleus and no elimination of chromatin could be demonstrated in later stages.

Tennent (1910) also succeeded in fertilizing sea-urchin eggs with sperm of different echinoderm classes. Thus the eggs of *Hipponoë* were fertilized with the sperm of *Ophiocoma* (brittle star) and of *Pentaceros* (starfish), and the eggs of *Toxopneustes* with the sperm of *Holothuria*. The method employed was to allow the eggs to stand two to three hours before adding the sperm. The development was highly abnormal in all cases.

Sea-urchin eggs have also been crossed with sperm of different phyla. Kupelwieser (1909 and 1912) has made a special study of this problem. He investigated the effect of the sperm of fourteen genera of mollusks and annelids on sea-urchin eggs, and obtained positive but usually scanty results in five cases, the others being negative. A high concentration of sperm and long exposure of the eggs was necessary. In all these cases membrane formation of the egg might also be induced by dead sperm or blood of the species. *Strongylocentrotus* ♀ × *Mytilus* ♂ gave the best results. The success of the fertilization seemed to depend on extract present with the sperm, which so affected the surface of the egg that one or more spermatozoa could enter. But if membrane separation occurred too rapidly, as a result of the sperm extract action, the sperm did not enter, and the eggs died. Once within the egg, if the condition was monospermic, events moved normally to a certain stage; an aster formed in association with the sperm nucleus; it then formed an amphiaster while the germ nuclei united. The male nucleus did not, however, form normal chromosomes and was eliminated; but the female formed its chromosomes, which divided in the usual way, and all nuclei were henceforward haploid and purely maternal. In a very small percentage of cases, development might proceed to the pluteus stage, which was usually defective. It was purely maternal as far as it went. In the very usual event of polyspermy the phenomena were essentially similar to dispermy or polyspermy within the species: aster formation from each sperm nucleus, a tetraster or polyaster first cleavage, abnormal development, and early death. The lack of specificity in the events between penetration and cleavage is thus clearly shown. Kupelwieser, however, erroneously concludes that all kinds of spermatozoa possess the same chemical stuff for activation of

The reader will note that these rapidly reviewed data on echinoderm hybridization fall into several groups, arranged according to the extent to which the egg reacts to foreign sperm as revealed by its subsequent development. Thus (1) in some hybrid combinations the eggs do not appear to react at all to foreign sperm; (2) in others, the foreign sperm may produce cortical changes without penetration; (3) the sperm may penetrate but perishes before union with the egg nucleus; (4) the sperm may unite with the egg nucleus with more or less elimination of male chromatin in the first or later cleavages; (5) the sperm chromatin may without elimination manifest its incompatibility by abnormalities in development.

It is obvious that the main difficulty with hybrid fertilization is in the cortical reactions. In the great majority of cases, apparently the foreign sperm is unable to call forth these reactions; in other cases, some component of these reactions is defective, e.g., the sperm may fail to penetrate, or the cortical reactions as a group may be quantitatively deficient. In most of the echinoderm crosses some artificial aid is needed to overcome this cortical bar; in general, these aids, such as increase of the alkalinity of the medium, or staling of the eggs, must be regarded as impairing the integrity of the cortex. One or more of the cortical factors in fertilization are thus highly specific.

In certain crosses, generally interspecific only, however, no artificial aid is needed. It nevertheless by no means follows that in such crosses species sperm and foreign sperm are equally effective in initiating development. In the only cases so far studied quantitatively—*Strongylocentrotus franciscanus* and *S. purpuratus* (Lillie, 1921*a*)—there is an enormous difference between the effectiveness of the species and the foreign sperm. More data are needed on the measure of specificity in fertilization between the reciprocals of each cross.

Once, however, any spermatozoön has crossed the cortical barrier, its reactions are no longer so highly specific. But it must be emphasized that during passage through the cortex, the spermatozoön reacts with the cortical substance. We have pointed out before that sperm which penetrate ova without initiation of the cortical reaction, e.g., in the case of *Arbacia* eggs in the presence of blood, cause no reaction in the interior of the ovum (cf. also the behavior of endoplasmic spheres, p. 479). The foreign sperm, no less than the species sperm owes its fertilizing effect within the egg to modification received in the cortex of the egg.

The elimination of chromatin in certain cross-fertilizations has been interpreted as due to the reappearance of a specific factor; however, it is equally possible to regard this phenomenon as a consequence of the treatment of the egg in removing the cortical block to cross-fertilization. Evidence for this point of view is to be found in the work of Wilson (1901), Gray (1913), and in some as yet unpublished work of Just. In all these cases eggs fertilized with *species* sperm showed abnormal nuclear behavior. Thus, Wilson treated fertilized eggs of *Toxopneustes* with ether, and obtained various grades of behavior of the germ nuclei. Gray treated fertilized eggs of *Echinus actus* with hypertonic sea water which caused elimination of chromatin. Just fertilized stale eggs (also eggs previously treated with dilute sea water) of *Echinarachnius* with species sperm, and obtained elimination of chromosomes undoubtedly of maternal origin as well as other aberrant behavior of the egg nucleus. Now, if in straight fertilization such phenomena obtain, it would seem improbable that similar phenomena in cross-fertilization should be due to the reappearance of a specific factor.

In Wilson's and in Gray's work the egg was subjected to ether or hypertonic sea water after fertilization with specific sperm; the initial reaction was completed and the sperm aster formed. The egg nucleus, on the other hand, had not yet come under the influence of the approaching sperm nucleus. It is true that the whole cytoplasm is affected as well as both nuclei, but the response to the treatment by the two nuclei cannot be the same since the physical condition of the nuclei is different at this time. The later behavior of the egg nucleus is due, therefore, to the disharmony between the nuclei which the treatment has brought about. Wilson's study very clearly lends itself to this interpretation. In the case of Just's observations on *Echinarachnius* eggs treated with dilute sea water or allowed to stale before insemination, there can be no doubt of the situation; the eggs alone have been treated. A spermatozoön entering such an egg reacts, forms an aster, and approaches the egg nucleus at a slower rate than in normal fertilization, much as in the *Toxopneustes* egg described by Wilson (1901). It seems reasonable to assume that if the egg alone is treated before fertilization the egg nucleus rather than the sperm nucleus is affected.

It is not necessary, therefore, to assume a specific factor as responsible for chromatin elimination in hybrid fertilization. The elimination may be merely the expression of disharmony between the egg nucleus and sperm nucleus, the tempo of their movements being altered because of the effect of treatment on the egg plasma before or after insemination. On this basis it is not at all unlikely that in some cases the female rather than the male chromatin is eliminated. Certainly, it is not necessary to postulate any theory of nuclear incompatibility to account for chromatin elimination. Moreover, the fact must not be overlooked that chromatin elimination is a normal process in many eggs.

Specificity in fertilization is undoubtedly due to a cortical block to foreign sperm. We have shown (p. 491) that the specificity of agglutination of spermatozoa by egg secretion (fertilizin) is of a similar order of magnitude to that of cross-fertilization; it is therefore natural to think of the cortical block in terms of agglutination. If the agglutination reaction is significant for releasing the cortical mechanism, as we suppose, the resistance to cross-fertilization becomes clear. But the breaking-down of this resistance by treatment with alkalies, or by staling the eggs, is a different problem, for such treatment certainly involves a partial loss of fertilizin. It is possible that with such partial loss is involved also a greater relative loss of another cortical substance that inhibits the cortical mechanism, much as the species blood has been shown to do (cf. p. 494). Such a hypothesis has no experimental basis, but it might possibly serve as guide in the investigation of this problem.

b) TELEOSTS

A great many experiments on cross-fertilization in teleosts have been made between species, genera, families, and orders. Newman (1908–15), for example, records seventy-eight heterogenic crosses between members of different families or orders of teleosts involving fourteen species; Moenkhaus (1904, 1910) records eighteen. Every cross-fertilization attempted was to some extent successful in that some, or even a large percentage, of the eggs segmented. Unlike the majority of echinoderm crosses, no artificial aids were used to secure the results. Even in the most distant heterogenic crosses, development might proceed to a late stage. Sooner or later, however, the heterogenic hybrids proved to be non-viable.

According to Newman, species fertilization succeeds more readily than any hybrid fertilization; the percentage of hybrid fertilization is always less under given conditions, and is frequently quite small. There is thus some evidence of specificity. The more ready union between gametes of the same species must depend upon some chemical relation between egg and sperm which is more highly developed between gametes of the same than between gametes of different species. This union obviously operates at the surface of the egg since the subsequent events of fertilization after penetration seem to proceed with equal facility whether the sperm belongs to the same or to different species. Moenkhaus, on the other hand, believes that in the case of teleosts studied by him there is no evidence of a specific adaptation of the egg for its own sperm. No adequate test of such a conclusion has been made. The dry method of insemination usually employed for teleosts exposes the egg to the highest possible sperm concentration and thus renders a quantitative examination of the problem of specificity impossible.

Clearly what is needed is a study of the optimum conditions for straight-fertilization together with a comparison of fertilization with foreign sperm under precisely the same conditions. That many marine teleosts spawn in fresh water, that the eel, for example, leaves fresh water to spawn in the sea, and that some marine teleost ova may be inseminated in dilute sea water reveal the possibility of a wide range in the adjustment of teleostean ova to surroundings. Moreover, the spermatozoa of different teleosts are not equally resistant. There may be thus several factors concerned in the normal fertilization of teleost eggs. In cross-fertilization these factors may constitute blocks which the method of dry insemination overcomes. It may well be, therefore, that the relative weakness of specificity in teleost hybridization is more apparent than real.

Following penetration, the spermatozoön in hybrid fertilization gives rise to the male germ nucleus whose behavior may be normal, i.e., without elimination of chromosomes (Moenkhaus, 1904; Günther and Paula Hertwig, 1914; and Morris, 1914). However, Pinney (1918), using the same species of teleosts that Morris used, reports elimination in the first and second cleavages, and no elimination in the reciprocals.

c) AMPHIBIA

Hybridization in Amphibia has been studied by several workers, including particularly Pflüger (1882), Born (1883, 1886), and Bataillon (1909); and more recently, the Hertwigs. Born found that except in the cases of *Rana fusca* ♂ × *Rana arvalis* ♀ and *Bufo vulgaris* ♂ × *Bufo cinereus* ♀ the hybrid eggs never develop to metamorphosis. Other combinations die at various stages. Viability as is usual in hybrid combinations is poor. Success of fertilization seems unrelated to systematic relationship. For example, even in species of the same genus fertilization may succeed one way and fail in the reciprocal. Thus Pflüger found that eggs of *Rana esculenta* fertilize readily with sperm of *R. fusca,* the eggs dying in the blastula stage; but the eggs of *R. fusca* never fertilize with the sperm of *R. esculenta. R. esculenta* and *R. arvalis*, however, fertilize reciprocally. Pflüger also found that eggs of *R. fusca* can be fertilized with the sperm of any other anuran. Bataillon found that the same is true of the eggs of *Pelodytes*; and that these eggs will fertilize with the sperm of the urodele, *Triton alpestris.*

According to Born a higher concentration of sperm is usually required for cross-fertilization than for straight. Born distinguishes three kinds of behavior of the gametes in cross-fertilization: (1) No reaction: e.g., *Bombinator igneus* and *R. esculenta*, reciprocal; *R. arvalis* ♂ × *R. fusca* ♀; *Pelodytes* ♂ × *R. arvalis* ♀. (2) Monospermic and normal fertilization: *R. esculenta* × *R. arvalis*, reciprocal; *R. fusca* ♂ × *Bufo cinereus* ♀. (3) Polyspermic fertilization and early death of the eggs: *B. cinereus* ♂ × *B. vulgaris* ♀; *Pelodytes* ♂ × *R. esculentus* ♀.

Certain experiments of the Hertwigs (1913) are not without interest, though it is doubtful if according to their interpretation they fall in the category of cross-fertilization. It was found that radium emanations injure spermatozoa, and that the radiation may be so graded as to leave the spermatozoa with ability to activate the eggs with no transfer of hereditary characters. Paula Hertwig (1913) showed that radiated sperm fail to take part in the formation of the zygote nucleus. This bore out O. Hertwig's previous assumption that such fertilization is a kind of experimental parthenogenesis: he had shown that in the cross *Salamandra* ♂ × *Triton* ♀ the eggs die in the blastula state, but if the sperm be strongly radiated the eggs produce larvae which possess the haploid number of chromosomes. This indicated that the egg chromatin alone was concerned in the development, and permits the inference that in hybrid fertilization with non-radiated sperm the early death of the egg is due to the multiplication of sperm chromatin. Günther Hertwig made a similar finding for the cross *R. fusca* ♂ × *B. vulgaris* ♀. On the other hand, Bataillon had previously shown that in the fertilization of *Triton* ♂ × *Pelodytes* ♀ the sperm nucleus takes no part in cleavage; nevertheless, the eggs die in the blastula stage.

On the whole, these data on cross-fertilization in Amphibia do not readily admit any single explanation. As a means of analyzing the problem of specificity in fertilization they therefore leave much to be desired. Fuller knowledge of the mechanism of fertilization in these forms is thus necessary for an explanation of the results here briefly reviewed.

B. SELF-FERTILIZATION

Having thus surveyed the work on hybrid fertilization we might next consider the data concerning the self-fertilization of hermaphroditic organisms, i.e., the fertilization of the eggs by the spermatozoa of the same individual. If dissimilarity of gametes is the cause that renders hybrid fertilization difficult, it might be expected that the closest possible relationship of gametes, which is found in hermaphroditic individuals, would involve the greatest compatibility of the gametes. But in appearance this is by no means always the case, for there are both hermaphroditic animals (rare) and plants in which self-fertilization is difficult or impossible.

The problem of self-fertilization has not been very widely investigated in the case of hermaphroditic animals, but sufficiently so at least to show that self-sterility is a rare phenomenon in the animal kingdom. In rhabdocoel Turbellaria reproduction by self-fertilization is common; it is also stated to occur occasionally in certain trematodes and cestodes in spite of an elaborate apparatus for cross-fertilization. Oligochaetes and pulmonates appear to reproduce exclusively by cross-fertilization; but Braun (1888) and Colton (1912, 1919) have shown that individuals of the pond snail *Limnaea* reared in isolation from the egg may produce fertile eggs. As parthenogenesis is unknown in mollusks it is almost certain that these eggs were self-fertilized. A. H. Cook reports a similar case for *Arion* (*Cambridge Natural History*). In the parasitic cirripeds (*Rhizocephala*) reproduction is invariably by self-fertilization (G. W. Smith, 1906), and the same is true of certain free-living nematodes (Maupas, 1900; Potts, 1910). In both of the latter groups special arrangements exist for insuring self-fertilization. Among the ascidians *Cynthia* and *Molgula* appear to be self-fertile, to a considerable extent (Morgan, 1904); but *Ciona* in the same group is self-infertile, at least to a considerable extent, which appears to vary somewhat for different localities and individuals (Castle, 1903; Morgan, 1904, 1905, 1910, 1923; Fuchs, 1914, 1915).

All these authors found certain individuals of *Ciona* in which the eggs are not susceptible of fertilization with the sperm of the same individual, although they may be fertilized with sperm of other individuals; and the sperm thus impotent on eggs of the same individual may fertilize perfectly the eggs of other individuals. The failure to self-fertilize in these cases is not due to immobility of the spermatozoa in the presence of own eggs or inability to penetrate the membrane of the egg (Morgan, 1923), but it is due to absence of the reaction that leads to actual fusion of the gametes. This incompatibility is by no means universal in *Ciona*, for all authors have found certain individuals in which self-fertilization may occur to a certain extent.

Thus Castle (1896) compared the percentages of fertilized eggs from isolated individuals with the percentages from pairs of individuals placed together. Observations were made on the same individuals for five successive days, and the fertilized eggs of each day were separately estimated. The result was that of fifty estimates from ten isolated individuals thirty-seven contained no eggs fertilized, nine from 4 per cent to 25 per cent fertilized, two contained 90 per cent of fertilized eggs, and in two cases no eggs were deposited. The paired individuals yielded twenty-five estimates, of which twenty-three showed 100 per cent fertilized, one yielded 20 per cent, and one none fertilized.

To this method of determining the extent of self-fertilization the objection has been made that spermatozoa of foreign origin may remain in the atrial cavity or tangled in the branchial basket, and give the effect of self-fertilization when none exists. Fuchs (1914, 1915), however, has shown that shed spermatozoa will not survive over twenty-four hours in sea water, so that tests are probably valid for determination after twenty-four hours of isolation, on the assumption that in the case of pairs both individuals shed their gametes.

To avoid this objection, artificial insemination has been practiced by the authors named. In such experiments Morgan finds an almost vanishing amount of self-fertilization; Fuchs, on the other hand, working at Naples, found that while in many cases no eggs segmented after self-fertilization, nevertheless *Ciona intestinalis* as a species is far from being completely self-sterile, though "a greater concentration of sperm is necessary to bring about any self-fertilization than would cross-fertilize 100 per cent of foreign eggs." Fuchs also determined that staling the eggs in sea water increases their susceptibility to self-fertilization up to a certain point; this was in marked contrast to cross-fertilization.

Morgan (1923) made a very significant contribution to this problem by showing that if the eggs of *Ciona* are removed from their membranes, and the test cells that lie between egg and membrane are likewise removed, they will then fertilize with their own sperm. Inasmuch as in self-fertilization of this form spermatozoa penetrate the membrane, the failure to self-fertilize must be ascribed to the test cells or to some substance produced by them. The block to self-fertilization in this case is thus analogous to that in self-sterile plants where the rate of growth of the pollen tube is slowed in comparison with not-selfed pollinations, so that the tube does not reach the egg cell. Thus in both cases the failure of self-fertilization does not lie in the gametes themselves, which puts the problem in quite a different category from that of hybrid fertilization.

C. DISCUSSION

If we gather together our account of specificity in fertilization it will be seen that the stage in which the phenomenon of specificity most commonly manifests itself, in the hybrid fertilization of echinoderms, teleosts, and Amphibia *is in the cortical reactions*. The various methods used to induce hybrid fertilization—

staling of eggs, high concentration of sperm, use of alkalies, etc.—have, therefore, this one feature in common, that they destroy the chemical or physical integrity of the cortex of the egg. If the cortical reactions and entrance of the spermatozoön are accomplished, there is no clear evidence of specific factors in the later stages.

The specific factor in the cortical reactions can hardly be of a purely physical character. We feel obliged to conclude that there is a chemical specificity more or less narrow, in the union of the gametes. The only phenomena in which we can so far detect a closer approach to this problem are those of specific sperm agglutination by egg secretions and specific inhibition of fertilization by the perivisceral fluid (cf. pp. 486, 489, 494).

III. THEORIES OF FERTILIZATION[1]

Boveri was the first to point to the necessity, in theories of fertilization, of making a sharp distinction "between the question how egg and spermatozoön produce a cell capable of division, and the question how these cells come to be capable of reproducing the qualities of the parent in the offspring." The latter problem has been answered by the behavior of the chromosomes in fertilization and by the chromosome theory of heredity; strictly speaking, only a small part of the chromosome theory is a problem of fertilization, for it involves the entire life-history and the succession of generations. The specific problem of fertilization is included in the first of Boveri's two questions concerning the union of the gametes to form a zygote.

Boveri's own theory of fertilization was, indeed, limited to the first of these questions. In his own words: "The ripe egg possesses all of the organs and qualities necessary for division excepting the centrosome, by which division is initiated. The spermatozoön, on the other hand, is provided with a centrosome, but lacks the substance in which this organ of division may exert its activity. Through the union of the two cells in fertilization all of the essential organs necessary for division are brought together; the egg now contains a centrosome which by its own division leads the way in the embryonic development" (Boveri, 1887, quoted from Wilson, 1895). This theory formed the center of a long controversy; it was undoubtedly the most influential point of view up to the beginning of the present century. We have already presented the evidences against it that have caused us to regard it now as of historic interest only (pp. 461–64).

It is to be noted that Boveri's theory was a theory of activation only; it did not take account of the problem of the union of the gametes, nor of the specificity in their behavior. It is to be noted also that it placed the initiation of activation too far forward in the series of events, after the completion of the cortical changes. Both of these defects are shared by those more modern theories which are based on parthenogenetic activation of the egg.

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[1] It is not possible to do justice to this subject in the brief space at our command. A somewhat fuller treatment is contained in Lillie's *Problems of Fertilization*, chap. vii. The other sources referred to in this chapter will enable the student to follow the subject still farther.

Students of experimental parthenogenesis have sought to find some common factor in the action of the effective agents responsible for activation. The list of agents that induce some degree of initiation of development is so various and long that there is a striking disagreement among writers both as to the common factor and as to the elementary effects that the agents produce on ova. By some it is held that the common effect of parthenogenetic agents is that of superficial cytolysis; by others, increased permeability; and by certain other workers, increased viscosity. All agents that cytolyze eggs or increase their permeability or increase their viscosity—varying according to the given author—are parthenogenetic agents. Valid objections may be raised to each of these notions as theories of experimental parthenogenesis quite apart from their application to a theory of fertilization.

In his book, *The Organism as a Whole* (1916), Loeb gives a convenient sketch of his cytolytic theory of parthenogenesis and fertilization (for earlier references see Loeb, 1913, and earlier references there). He holds that the essential feature in the activation of the egg, whether by fertilization or by parthenogenesis, is the change underlying membrane formation, which he conceives to be cytolysis of the superficial layer of the egg. His reason for the conclusion is that "all those substances and agencies which are known to cause cytolysis or hemolysis will also induce membrane formation": (1) fatty acids; (2) saponin or solanin or bile salts; (3) lipoid solvents, e.g., benzol, toluol, ether, chloroform, etc.; (4) bases; (5) hypertonic or hypotonic solutions; (6) rise in temperature; (7) certain salts, e.g., $BaCl_2$, $SrCl_2$, NaCNS; (8) the blood serum or cell extracts of certain foreign species. In the sea urchin the development does not proceed to cleavage by action of the single agent, except in the case of hypertonic solutions, but a second agent is required to bring about further development. Hypertonic sea water is the second agent most commonly employed; this, when used for the proper length of time, insures subsequent normal development. Loeb therefore states that the action of the first agent leaves the egg in a sickly condition, and the action of the second agent is required to save the life of the egg. It is a corrective agent remedying an unavoidable excess of action of the first agent. Similarly, he held that the sperm carries both a lysin and a corrective agent active in the fertilization of the egg.

There is abundant evidence to show that in experimental parthenogenesis of sea urchins two agents are unnecessary; the so-called corrective factor alone will suffice. Thus hypertonic sea water used alone is capable of initiating cell division in the eggs of sea urchins, as Morgan (1896, 1900) long ago discovered, and as many other workers have subsequently confirmed. Loeb's first method for the experimental production of plutei from uninseminated eggs consisted in the hypertonic treatment only. In most examples of artificial parthenogenesis, indeed, one agent suffices for the initiation of development. Moreover, cytolysis, strictly speaking, is a wholly different phenomenon from that initiated in the process of membrane separation (see Just, 1920, 1922*b*, 1922*c*). The "cytoloysis" of an egg that has had an overexposure to the agent of membrane separation, and that of an egg that has the proper exposure to induce membrane lifting, spring from different causes (cf. Herlant, 1918; and Just, 1919*c*). The application of the conception of superficial cytolysis to actual fertilization has not been substantiated; the postulate of this conception that sperm carry a lysin effective in fertilization of the egg has received neither logical nor experimental support. For these reasons, among others, we cannot admit that Loeb's conception, though it was a powerful stimulus to research, contains a workable hypothesis of activation.

An increase of permeability of the egg has also been held by other writers (especially R. S. Lillie, 1913*a*, 1913*b*, and later papers) to be an underlying cause of the initiation of development. Changes in this sense following activation by artificial agents and by sperm have been determined by measurements of various kinds (see p. 500). These have been made, however, so long after the actual initiation of the cortical changes, that the changes actually found must be interpreted as a *result* not the *cause* of activation. R. S. Lillie, who has especially emphasized the significance of increase of permeability in activation processes, holds (1915) that the fundamental "releasing" process underlying activation of the egg is probably some change of the nature of degelation or decrease of viscosity in the cortex of the egg which presumably allows substances to come together and to interact which in the condition of the cortex of the unfertilized egg are kept apart. Loeb (1916) advocates also a fundamentally similar point of view with the difference that he appeals to cytolysis as the releasing factor.

According to other authors the cause of activation lies in an increased gelation of the cytoplasm. A rhythm of viscosity changes in developing ova was demonstrated many years ago by Mrs. Andrews (1897) by means of pressure experiments. Other workers employing different methods have demonstrated changes in the consistency of the egg protoplasm during fertilization (cf. p. 502).

Heilbrunn, Chambers (1917), and Seifritz (1920), though not wholly in agreement, find evidence of changes in the viscosity of marine ova after fertilization related to mitosis. But there is no good evidence that this increased viscosity is the cause of activation as postulated by Delage (1908, 1913), Fischer and Ostwald, and Heilbrunn. Says Heilbrunn (1915):

> The view that I would maintain is that the only physico-chemical effect which all parthenogenetic agents possess in common is the production of a gelatinization or coagulation within the egg. Hence, I regard this gelatinization (or coagulation) as the direct cause of the initiation of development. Leaving theory aside, it is possible to demonstrate that all parthenogenetic agents actually do produce gelatinization or coagulation within the egg.

But here again the "cause" does not precede the initiation of development, but comes after the cortical changes are completed.

According to Glaser (1914), sea water rich in egg secretion is capable of initiating development in eggs of *Arbacia*. This phenomenon he calls "auto-parthenogenesis." The writers have been unable to obtain this result unless the eggs are left uncovered during exposure, so that the medium becomes decidedly hypertonic; in such a case the hypertonicity rather than increase of egg secretion should be held responsible, especially as the same result may be obtained without increase of egg secretion.

Miss Woodward (1918) and Glaser (1921*a*, 1921*c*) believe that the phenomena described by the writers as due to fertilizin are really due to two distinct substances which may be isolated from egg secretion by appropriate chemical treatment. The one of these is a parthenogenetic agent, the other a sperm agglutinin. The former has fat-dissolving properties, and is assumed to be a lipase, for which the name "lipolysin" was adopted.

A theory of activation was proposed by Miss Woodward (1918) in the following terms: "The resting egg cell contains enzymes which control metabolism, unsaturated fatty acid which inhibits enzyme action, and lipolysin which reacts with the unsaturated fatty acid to make it innocuous." Activation is caused by any method that increases the ratio of activating enzymes to fatty acid, such as increase of lipolysin.

According to Miss Woodward lipolysin is a fat-splitting enzyme; Glaser (1921) substantiates this claim. Lipolysin then splits fats and so increases the amount of fatty acid. This would seem to introduce a logical fallacy if the first step in activation is conceived as the reduction of the fatty acid inhibitor. The theory is not consistent in all of its parts. The method employed for obtaining lipolysin is calculated to yield split products at stages in the preparation. The fact that it so closely resembles methods used in treating mammalian sera for ovocytase (Robertson) and for thrombin is perhaps significant, since extractives from such blood can initiate changes in echinoderm ova.

No single theory can account for all the phenomena of fertilization as we have defined it. The minimum requirements of a theory of fertilization are to account for the specific union of the gametes and resulting activation of the ovum. A theory of activation alone does not meet both requirements; the fertilizin theory (Lillie, 1914) aims to do so. It postulates that a substance borne by cortex of the egg (fertilizin) exerts two kinds of actions, (1) an agglutinating action on the spermatozoön, and (2) an activating effect on the egg. The spermatozoön is conceived, by means of a substance that it bears (agglutinable substance or "sperm receptors") and which enters into union with the fertilizin of the egg, to release the activating effect of the fertilizin within the egg. These substances are conceived as linked in line thus: sperm receptors—fertilizin—egg receptors, and not directly as sperm receptors $\left\langle\begin{matrix}\text{Fertilizin}\\ \text{Egg receptors}\end{matrix}\right.$

because the sperm receptors are able to bind the fertilizin in the absence of egg receptors, but are unable to bind the egg receptors (as shown by failure of activation) in the absence of the fertilizin. This theory as thus outlined ignores, provisionally, the physical conditions shown to arise in connection with fertilization which have been so carefully studied by Loeb, R. S. Lillie, Heilbrunn, and others, but no contradiction is involved.

The data on which the theory rests have been outlined in many places in this section. Certain criticisms have been discussed in Lillie's *Problems of Fertilization* (1919). In conclusion let us note how the theory fits the main principles of fertilization. In the first place, it is consistent with the major thesis that the egg is an independently activable system; whether the fertilizin is activated by the spermatozoön, or in some other way, should make no difference, except in a quantitative sense in certain cases, in the development of the egg. In the second place, it explains the association of activation of the egg with fertilization. In the third place, it explains the non-fertilizable condition of already fertilized eggs, because, as has been noted (p. 491), all free fertilizin is bound in some way shortly after fertilization, and fertilized eggs produce no more of it. In the fourth place, it explains why spermatozoa are inert in immature eggs, in parts of eggs devoid of cortex (p. 479), and in eggs activated by parthenogenetic agents (p. 502). In the fifth place, it explains the formation of the sperm aster by virtue of the necessity for the sperm to pass through the cortex of the egg, becoming fertilized there, before it can induce aster formation (p. 464). It is also consistent with the facts of merogonic fertilization. Finally, it furnishes a basis for understanding the problem of specificity, because the agglutination phenomenon exhibits comparable specificity, as we have seen (p. 491).

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SECTION IX

CELLULAR DIFFERENTIATION

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CELLULAR DIFFERENTIATION

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I. PRINCIPLES OF DIFFERENTIATION

1. *Definitions and terms:*

All differentiation is transformation from a more general and homogeneous to a more special and heterogeneous condition. Dedifferentiation, on the other hand, is the reverse of this. Many differentiations are purely temporary, others are more permanent, but, in general, protoplasm is not constant or static with respect to differentiation, but oscillates between different phases. These oscillations or cycles may be short as in physiological processes of assimilation and dissimilation, contraction and expansion, mitosis and intermitosis; or they may be long as in life-cycles of individuals, in which differentiation may be more or less permanent and irreversible. These life-cycle differentiations are usually known as *development,* which may be defined as progressive differentiation, co-ordinated as to time and place. It is these developmental differentiations to which attention is especially directed in this section. Differentiation is always associated with integration; specialization with co-ordination and co-operation. These two are correlative processes, two aspects of one thing, namely, organization. Indeed, organization was defined by Herbert Spencer as differentiation and integration. In this section, attention must be focused largely on the former, but the two are inseparable in living organisms.

Differentiation has often been defined as "physiological division of labor," but it is also "morphological division of substance," for these are merely two aspects of one and the same thing, and in life they are actually inseparable. Consideration of the processes of differentiation are chiefly physiological; of the results, morphological; but in this case, as in that of most other vital phenomena, it is not profitable or even possible to distinguish sharply between these two methods of study. This is especially true in dealing with protoplasm and cells, and accordingly these two aspects of differentiation will be considered together.

2. *Causes of differentiation:*

a) CHEMICAL AND PHYSICAL

In the last analysis all differentiations are probably the results of chemical changes in protoplasm, although there are differentiations due to chemical changes in metaplasm and intercellular substances; however, the peculiar constitution of these formed substances, which makes possible certain differentiations in them, is in turn due to specific activities of the protoplasm by which they are formed, so that in the last possible analysis we find all differentiations the results of chemical changes in protoplasm. In this section no attempt is made to deal with these chemical changes but consideration is limited largely to processes which are visible under the microscope—to molar rather than to molecular or atomic phenomena. It is important to realize that the ultimate causes of differentiation must be found in chemical and physical phenomena, but it is equally important to remember that the last step in such an investigation cannot be taken before the first and intermediate steps, and the first step is the study of morphological and physiological differentiation under normal and experimental conditions.

b) MORPHOLOGICAL AND PHYSIOLOGICAL

The causes of differentiation, as well as of all other vital phenomena, may be classified as intrinsic and extrinsic, or as the reactions of (1) the living substance to (2) environmental stimuli. Overemphasis on either one or the other of these factors leads to confusion and to false conclusions, and there is certainly no room for views which exclude either intrinsic or extrinsic causes. Both are indispensable in differentiation or in any other life-process. There is no such thing as "self-differentiation" in the sense that extrinsic causes are excluded, nor "dependent differentiation" in the sense that intrinsic causes are non-operative. The only question that can properly be raised is as to the relative importance of each of these factors in any instance; but this question has afforded ground for much controversy. Much of this controversy might have been avoided if only opponents had always recognized that the question was merely one of the relative importance to be ascribed, in any case, to these two classes of factors. But the epigenesists overemphasized the importance of external stimuli and the position, connection, and interaction of parts, while the endogenesists fixed their attention too exclusively on the internal organization of the protoplasm.

This same tendency to exclude either intrinsic or extrinsic factors is seen in discussions regarding theories of heredity and evolution, as well as of differentiation and development. It is sometimes reflected in a sort of hostility between morphologists and physiologists as well as in the common and fundamental error of attempting to separate structure and function. The cytologist is particularly liable to overemphasize the intrinsic factors of protoplasmic and cellular organization since he must deal largely with fixed and stained preparations and only to a limited extent with the reactions of this living organization to external stimuli. On the other hand, the physiologist is prone to overemphasize the extrinsic factors and to overlook or minimize the microscopic organization of the cell. But structure and function are actually inseparable in a living thing, they are merely two aspects of one reality; intrinsic and extrinsic causes are inseparably united in all life-processes, and both are involved in all differentiation, development, and evolution.

3. *Processes of differentiation:*

Two fundamental processes are concerned in differentiation, (1) the *formation* of unlike structures with specific functions and (2) their *segregation* in different parts of the cell or organism. It is a serious error to suppose that differentiation consists merely in the sorting and isolation of multitudinous substances and activities already present in the germ cells at the beginning of development; such an error is akin to the old doctrine of preformation. On the other hand, it is an equally serious error to conceive that new structures and functions of *extrinsic* origin are added to the cell or organism in the course of its development, or that differentiation consists in the addition of foreign organization to the developing organism. In reality, differentiation is *transformation* of general structures and functions already present into more special structures and functions, under the influence of environmental stimuli. In this process new structures and functions are formed by the transformation of old ones and not by extrinsic additions. After the fertilization of the egg no particle of living substance is added to the developing organism; matter and energy are added as food or raw materials which are then assimilated and elaborated by the living substance into its own organization, but foreign organization is not incorporated as such. On the contrary, development is from within and not from without. The germ is a living thing, separate and distinct from all others, and development is one of its functions, just as are also assimilation and growth.

In all differentiation there is not only the formation of special structures and functions from general ones, but there is also the *segregation* and *isolation* of these in different parts of the cell or organism. When once segregation and isolation of particular structures and functions have taken place, a new basis for further differentiation is provided, so that progressive differentiation is dependent upon not only the formation of new substances but also upon their isolation.

II. UNIVERSAL DIFFERENTIATIONS OF CELLS

Protoplasm exists only in the form of cells, and all cells have certain fundamental differentiations. Indeed, protoplasm is a morphological and physiological and not a chemical concept. Although it is customary to speak of "undifferentiated" protoplasm and cells, such things do not really exist; what is always meant by such a term is "less differentiated" as contrasted with "more differentiated." The most general and evident differentiations of the cell are into nucleus and cell body, of protoplasm into *karyoplasm* and *cytoplasm.*

1. *The nucleus:*

The most readily distinguishable portion of the karyoplasm is the *chromatin,* which usually has a strong affinity for basic dyes, and which exists in the form of granules or definite morphological bodies known as *chromioles.* There are at least two different forms of chromatin, the *oxychromatin,* which stains relatively faintly, and contains relatively little nucleic acid, and the *basichromatin*, which contains more of this acid and stains deeply; the basichromatin certainly gives rise to oxychromatin, and perhaps the latter may be transformed into the former. The nucleus usually contains other substances which do not stain with basic dyes and which are known collectively as *achromatin;* among these are the nuclear sap or *karyolymph* and the *linin*, or the more solid part which holds the chromioles in a definite relation to one another and which is the main form-conserving part of the nucleus.

Usually these nuclear constituents are inclosed within the *nuclear membrane* which separates them from the rest of the cell contents, but there are some unicellular organisms in which they are not organized into a single nucleus distinct from the cell body, but exist as minute bodies scattered through the cell, each surrounded by its own membrane or film. Even the single nucleus which is found in most cells is composed of many smaller nuclear or chromosomal vesicles, as may be seen in the formation of daughter-nuclei following mitotic division (Fig. 6 *E*, *F*, p. 564).

2. *The cytoplasm:*

The cytoplasm also consists of several different constituents and most of the differentiations which occur in development are located in the cell body. There is present almost universally a clear, non-granular layer of *hyaloplasm* at the periphery of the cell, known as *ectoplasm*, and a granular central portion, the *endoplasm.* The former is usually more dense than the latter, and in some cases portions of the hyaloplasm extend through the endoplasm and hold the nucleus in a more or less definite position with reference to the ectoplasmic layer.

In addition to the constituents named, cytoplasm contains various "inclusions," such as water, oil, yolk, pigment, granules, mitochondria, plastids, and various products of differentiation. To what extent these are essential parts of the cytoplasm is not clear in every case; many of them are evidently the products of protoplasmic activity or are foreign bodies imbedded in the cytoplasm; however, they are frequently of great importance in differentiation.

3. *Centrosome, aster, and sphere:*

Another structure which is generally found in the cell body is the *centrosome*, though it is apparently absent in higher plants and in some animal cells. It sometimes comes out of the nucleus (Schockaert, 1901; Boveri, 1901) and in form and nature resembles the achromatic portion of a nucleus or of a chromosomal vesicle, so that R. Hertwig (1895, 1899) has called it "a nucleus without chromatin"; a somewhat similar view has been expressed by Heidenhain (1894), Boveri (1901), Conklin (1902), and others. In some cases the centrosome is surrounded by a membrane during resting stages which dissolves at the beginning of mitosis, as is true of the nuclear membrane (Boveri, 1901; Conklin, 1902). In stages of mitosis, a central granule within the centrosome,

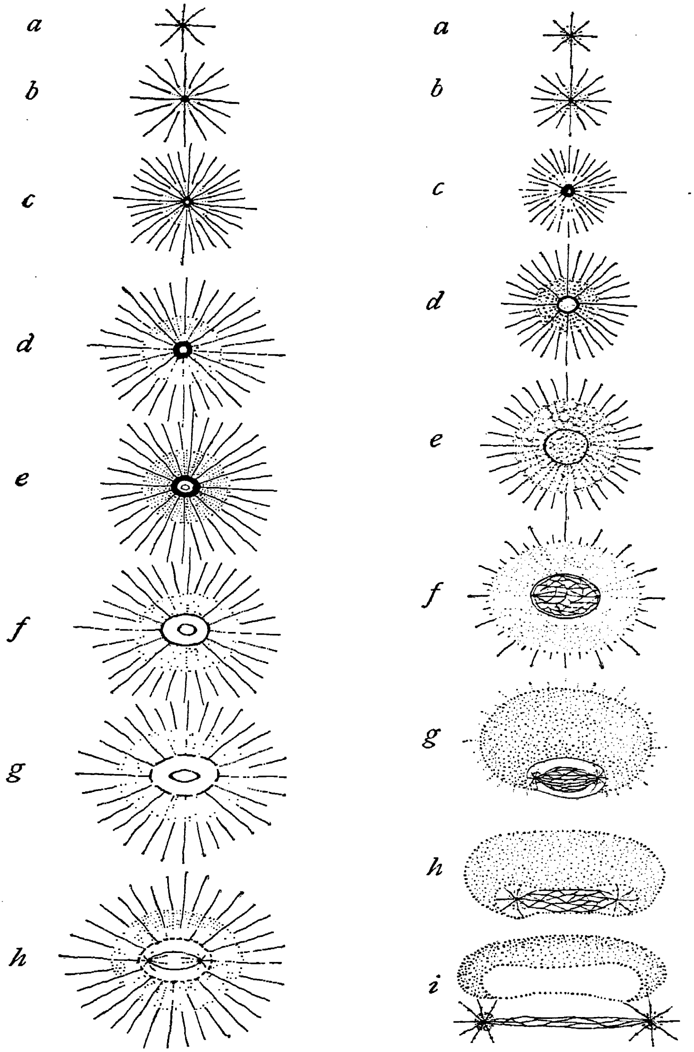

Fig. 1.—Successive stages of centrosome and sphere in *Crepidula*. Left-hand column, maturation divisions; right-hand column, cleavages. The small granule at the center of the rays (*a*) grows to a hollow sphere (*d*), within which one or more granules give rise to the netrum or initial spindle (*g* and *h*) which then moves out of the surrounding sphere substance (*i*).

the *centriole*, divides and gives rise to a minute spindle, the *netrum* (Boveri, 1901), which then moves out of the old centrosome, and the latter disappears in the sphere surrounding the centrosome and ultimately in the cytoplasm. In all of these respects the centrosome resembles "a nucleus without chromatin" (Fig. 1).

In stages of cell division the centrosome is surrounded by radiations known as the *aster;* this consists in part of hyaloplasm from the cell body and of achromatin from the nucleus, and its substance is probably identical with the *archoplasm* of Boveri (1888). It gives rise to the portion of the mitotic spindle lying outside the nucleus as well as to the astral radiations. The intranuclear portion of the spindle arises within the nuclear area after the nuclear membrane has broken. In some cases (*Crepidula*, *Styela*) it is evident that these intranuclear spindle fibers grow at the expense of granules of oxychromatin (Figs. 15, 16); in all cases they are connected with the chromosomes, and their further growth is dependent upon these. Where centrosomes are lacking, as in the higher plants and in the maturation divisions of certain animals, the intranuclear spindle is the only portion present. Where both extra and intranuclear portions of the spindle are present, they are usually intimately united, but in the eggs of *Ascaris* and ascidians these two portions can be distinguished and can even be separated by centrifugal force in certain stages of mitosis. Probably, therefore, oxychromatin and centrosomes may cause the formation of fibers of similar character.

During resting stages the substance of the aster is condensed into a rounded body, surrounding the centrosome, which is known as the *astrosphere*, or simply the *sphere*. Isolated portions of archoplasm, or of achromatin, when scattered through the cell, may give rise to radiations known as *cytasters* (Wilson, 1901). Cytasters never form in an egg while the germinal vesicle remains intact; after the dissolution of the nuclear membrane and the escape of a relatively large quantity of nuclear sap, cytasters may appear, during division stages, wherever isolated portions of this escaped nuclear material are found (Conklin, 1902, 1912*c*). In *Crepidula* there are no true centrosomes in these cytasters, they do not undergo division, but on the contrary tend to fuse together, and they do not take part in the division of the chromosomes (Fig. 2). On the whole, centrosomes, asters, and spheres seem to partake of the nature of both nucleus and cytoplasm and to be more or less intermediate between the two.

4. *Origin of these differentiations:*

Some of these general differentiations of the cell are temporary and appear and then disappear with certain phases of cell activity. For example, chromosomes, asters, and spindles differentiate during mitosis and dedifferentiate during intermitosis. Chromosomes give rise to chromosomal vesicles and vesicular nuclei, and these in turn give rise to chromosomes; oxychromatin transforms into basichromatin, and basichromatin into oxychromatin, etc.

It is usually said that the nucleus is a permanent organ of the cell, and there has been much discussion as to whether the centrosome is also a persistent organ. But in cells undergoing mitotic division neither the vesicular nucleus nor the chromosomes as such are permanent; the larger part of the resting nucleus is drawn from the cell body during intermitosis and goes back into the cell body during mitosis. The differentiation of the entire nucleus is not permanent; nevertheless, it is evident that there is something which persists and undergoes cyclical transformations during all these phases of division. The centrosome also undergoes transformations during the phases of mitosis and intermitosis, and the question as to whether it is a permanent cell organ, in the sense in which the nucleus is, depends upon whether there is anything which persists and undergoes cyclical transformations. Evidently, few if any of these visible differentiations of cells are permanent; the things which do persist and undergo these transformations must be units or bodies of a smaller order than these larger structures. In the case of the nucleus it seems probable that chromioles and some connecting substance such as linin do persist through all the phases of the division cycle; if so, these represent permanent differentiations of the cell.

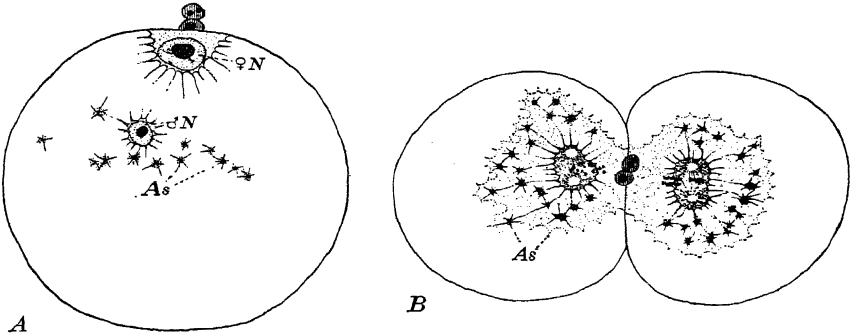

FIG. 2.—Cytasters (*As*) in the egg of *Crepidula* treated with 2 per cent NaCl four hours; diffused nuclear substances from germinal vesicle (archoplasm) condensed into cytasters.

5. *Cell polarity and symmetry:*

Another important cellular differentiation consists in the relative positions in a cell of its various constituents, that is, of the manner in which its different parts are integrated. Some of the more general differentiations of this type may be classified as polarity, symmetry, and pattern.

Polar differentiation, or more briefly polarity, may be defined as the condition of having unlike poles, particularly in the chief axis of a body, while symmetry is the condition of having like poles in certain cross-axes. The polarity or symmetry of an entire organism, or of any of its parts, is an expression of the relative positions of subordinate parts with respect to the chief axis and the cross-axes; in this sense it is customary to speak of the polarity and symmetry of organisms, organs, cells, nuclei, etc.

It has been known for a long time that cells in general show polar differentiations. The chief axis of a cell was called by Van Beneden (1887) its "organic axis," by Heidenhain (1894) the "cell axis," and by the latter it was defined as the axis passing through the centrosome and the nucleus of the resting cell. However, it is evident that there is a polarity of the cell body more or less independent of the positions of nucleus and centrosome as is indicated by the positions of other structures and substances within the cell, such as aggregations of ectoplasm, spongioplasm, mitochondria, fibrils, granules, yolk, pigment, and other inclusions. The resting nucleus also shows polar differentiation as was pointed out long ago by Rabl (1885), the pole which lies nearest the centrosome being called by him the "Pol" or central pole, the opposite being the "Gegenpol" or distal pole. Within the nucleus the chromosomes in the early prophase of mitosis are usually more closely aggregated at the central pole. The centrosphere, that is, the large centrosome of the resting cell, also has a polarity of its own, its chief axis being that in which the daughter-centrosomes move apart and form the initial spindle. This initial spindle, or "netrum" (Boveri, 1901) (Fig. 1 *h*, *i*), usually lies at right angles to the preceding spindle axis. But the axis of the fully developed spindle may differ from that of the initial spindle since there are characteristic movements of the cytoplasm which transport the spindle into its final position. During the last phase of nuclear division ("telophase" of Heidenhain, 1894) the daughter-centrosomes and nuclei, in the case of epithelial cells and blastomeres, turn back toward the original axis of the cell body, the centrospheres remaining attached to the nuclei at their central poles and moving to a point on the free surface of the cell which is nearest to the original cell axis. The axes of the daughter-cells which are thus formed are approximately parallel to the old cell axis (Fig. 15 *D*). The axis of the cell body does not change every time the centrosomes separate in division, but it remains relatively constant while the mitotic figures may form any angle with it, but at the close of division the centrosomes and nuclei come back once more into the chief axis of the cell body. Thus the axis passing through the nucleus and centrosome coincides with the axis of the cell body only during the resting stage of the cell. The cytoplasmic axis is more persistent than the axis of the nucleus, centrosome, or mitotic figure, and in the main it dominates all the others; consequently it will be referred to as the "cell axis."

Although the cell axis is usually marked out by the position of the resting nucleus and centrosphere, it is not dependent upon that position. By pressure or centrifugal force the positions of nucleus and centrosphere may be changed without permanently altering the cell axis, as is shown by the fact that these structures usually come back once more to their normal positions as soon as the pressure is removed (Fig. 3 *E*, *F*). Also the position and direction of the mitotic

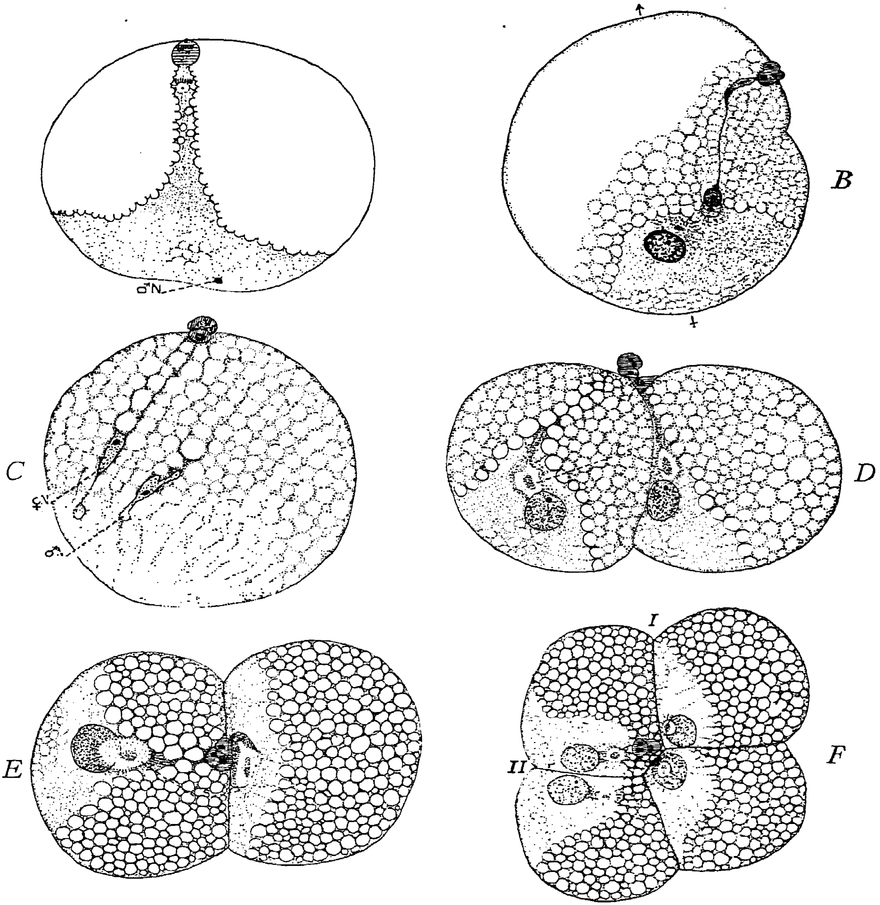

FIG. 3.—Centrifuged eggs of *Crepidula*. *A*, Cytoplasm centrifuged away from animal pole leaving a lane of cytoplasm connecting with maturation spindle. *B*, Centrifuged during the second maturation division; spindle greatly elongated; nuclei proportional in size to volume of cytoplasm in which they lie. *C*, Cytoplasm centrifuged away from animal pole leaving strands of spongioplasm between yolk spheres; egg and sperm nuclei stretched out of shape. *D*, Centrifuged in two-cell stage; spheres lie between nuclei and animal pole, connected with both by strands of spongioplasm. *E*, Two-cell stage centrifuged at right angles to preceding but showing similar results. *F*, Four-cell stage centrifuged in same axis as *E*, showing strands of spongioplasm connecting spheres with nuclei, on the one hand, and with ectoplasm at animal pole, on the other.

figure and of the resulting division plane may be changed experimentally without changing the real cell axis. In fine, the cell polarity persists in the organization of the cytoplasm after the positions of centrospheres, nuclei, mitotic figures, and cleavage planes have been changed. But while in such cases the polarity of the cell persists in the cytoplasm, there is evidence that the polarity of the latter has developed in connection with and in definite relation to the polarity of the nucleus and centrosome.

6. *Interchange between nucleus and cell body:*

a) DURING INTERMITOSIS

There are many evidences of an interchange of substances between the nucleus and the cytoplasm. Of course the growth of a nucleus must be accompanied by the intake of something from the cell body. The entire nucleus, and even the individual chromosomal vesicles, are surrounded by some sort of a membrane. Furthermore, it is evident that while this membrane is intact it does not permit the passage of formed bodies, but only of fluid with substances in solution. During the entire period from the anaphase of one mitosis to the prophase of the next, there is an inflow of fluid into the nucleus carrying in substances in solution which contribute to the growth of the chromatic and achromatic portions of the nucleus. It seems probable that the substances thus carried in are not chromatin granules or large colloidal aggregates of molecules but rather substances composed of relatively small molecules or aggregates which will pass by osmosis through the nuclear membrane. Probably neither chromatin nor linin enters as such but rather these are built up inside the nucleus from substances which are able to pass through the nuclear membrane, as is the case, for example, in the absorption of food from the alimentary canal. Substances that stain like chromatin are often found in the cytoplasm, and Danchakoff (1916) has described a "basiphilic chromatic substance" in the cytoplasm of the starfish egg which is said to be taken into the nucleus and to contribute to the growth of the nuclear chromatin. If this occurs, it must be assumed that it passes the nuclear membrane not as chromatic granules but in solution. Masing (1910) found that there is no perceptible increase in the nucleic acid content of the egg of *Arbacia pustulosa* between the 1-cell stage and the blastula, although he claims that there is an increase of about one thousand fold in the nuclear mass of the egg.[1] Therefore, he concludes that the nucleic acid of the nuclei must be drawn from a preformed stock in the egg plasma, which, he says, does not increase in volume during this period. If this is true, nucleic acid must pass from the plasma into the nucleus, and it is possible that many other organic substances may enter through the nuclear membrane by osmosis.

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[1] This is certainly an error. In *Crepidula* the volume of all the nuclei at the 70-cell stage is barely equal to that of the germinal vesicle just before the first maturation division, while in *Styela* the volume of all the nuclei at the 256-cell stage is only about one-quarter that of the germinal vesicle. Since the germinal vesicle is unusually large, and since egg and sperm nuclei at the beginning of the first cleavage are rather variable in size, it would probably be fairer in estimating the growth of the nuclei during cleavage to start with the maximum size of the nuclei at the 2-cell stage. From the 2-cell to the 70-cell stage of *Crepidula* the total nuclear volume increases only 2.24 times. In *Styela* from the 2-cell to the 256-cell stage it increases only 4.52 times. This is far short of "a thousand fold," and is not even a "colossal

On the other hand, many investigators have described the escape of nuclear substances from the resting nucleus into the cell body, either by exosmosis or through breaks in the nuclear membrane. In particular, R. Hertwig and his pupils have studied this phenomenon. In the case of *Actinosphaerium*, Hertwig (1902) observed the escape of chromatin granules into the cell body where they constitute what he calls *chromidia;* during this process the nucleus itself may decrease in size and even disappear. He also observed the escape of chromatin granules from the germinal vesicle of metazoan eggs, as well as from the nuclei of certain somatic cells. These observations have been confirmed and extended by Goldschmidt (1907), Goldschmidt and Popoff (1907), and others. In these cases the nuclear membrane breaks and thus permits the escape of formed bodies into the plasma. Schaxel (1911) also has described in the oöcytes of several animals the emission of chromatin from the nucleus, apparently as formed bodies, through breaks in the nuclear membrane. He summarizes (1915, pp. 29, 33) his observations on the interaction of nucleus and plasma in the formation of the egg, as follows: (1) There is first the growth of the nucleus, formation of nucleolus, and increase of chromatin ("chromasie of nucleus and achromasie of plasma"). (2) Then follows chromatin emission from the nucleus and increase of cytoplasm ("achromasie of nucleus and chromasie of plasma"). A further discussion of *chromidia* is given by Cowdry.

In spite of these and many other observations of the escape of formed bodies through breaks in the nuclear membrane, it is open to question whether these substances may not pass through the nuclear membrane in solution, and then be reconstituted in the plasma. In general, the nuclear membrane remains intact during intermitosis, and interchange between nucleus and cell body takes place by osmosis through this membrane.

During great functional activity the nucleus often shrinks and becomes irregular in outline, whereas it again becomes full and spherical during periods of inactivity. Hodge (1892) observed such changes in the nuclei of nerve cells, and others have seen them in many gland cells. For example, in the liver cells of *Crepidula* the volume of the nucleus, in a cell filled with secretion products, is only about one-quarter as great as in a cell without secretion. This can only

growth." With reference to Massig's statement that plasma does not increase in volume from the 1-cell to the blastula stage, it must be said that this applies only to the entire cell contents; the cytoplasm does increase at the expense of the yolk during all this period. In *Crepidula* the volume of cytoplasm more than doubles between the 1-cell and the 24-cell stage and the yolk decreases in volume by nearly one-half. A similar condition is found in *Lymnaea*, *Physa*, *Planorbis*, and probably in all eggs that contain a considerable amount of yolk.

mean that substances pass from the nucleus into the plasma during periods of great functional activity and in the reverse direction during periods of rest.

b) DURING MITOSIS

But although this interchange usually takes place by osmosis through the nuclear membrane during periods between mitoses, with the disappearance of the nuclear membrane during mitosis, formed bodies are liberated from the nucleus into the cell body. In dividing cells of Metazoa and Metaphyta, the osmotic inflow into the nucleus occurs from the end of one mitosis to the beginning of the next, when it suddenly alternates with an outflow of nuclear material into the cytoplasm brought about by the dissolution of the nuclear membrane. This alternate growth and collapse of the nucleus has been called nuclear "diastole" and "systole" (Ryder, 1894). Microchemical tests show that the substances that are released into the cell body with the disappearance of the nuclear membrane are not the same as those which entered during its growth. Not only fluid nuclear sap is thus released, but also many formed bodies such as chromosomes, nucleoli, oxychromatin granules, and linin.

In the ascidians, *Styela* and *Ciona*, the volume of the germinal vesicle just before maturation is more than one hundred times the volume of the first maturation spindle; all of the nuclear contents except this small portion represented by the spindle become a part of the cytoplasm. Even the oxychromatin which escapes into the cell body at mitoses is often more abundant than that which goes to form the chromosomes; Gardiner (1898) estimated that in the first maturation division of the egg of the flatworm, *Polychaerus*, five hundred times as much chromatin was discharged into the cell as went to form the chromosomes. Similar conditions are found in many other eggs, and probably in all cases this "emission chromatin" from the germinal vesicle is more voluminous than the basichromatin of the chromosomes. This emission chromatin may be partially or wholly dissolved in the nuclear sap before its escape into the cell body, or it may escape as granules which later undergo solution. In *Crepidula*, this chromatin is largely dissolved in the nuclear sap which then stains with basic dyes and may be identified in the spindles and asters, and probably in the chromatic granules which are widely scattered through the cytoplasm following mitosis. This distribution of chromatic granules through the cytoplasm is especially evident in certain eggs.

In this connection one recalls also the dissolution of the macronucleus in the conjugation or the endomyxis of ciliate Infusoria. In the ordinary divisions of these Protozoa, the nuclear membrane remains intact in both the macronucleus, which divides by amitosis, and the micronucleus, which divides by mitosis. Consequently, in these divisions there is no such escape of nuclear substances into the cell as takes place in Metazoa during mitosis. But periodically, during conjugation, and also in endomyxis (Woodruff and Erdmann, 1914), the entire macronucleus disintegrates and is dissolved in the cytoplasm.

In this respect the macronucleus of the Ciliata resembles the achromatin and oxychromatin of metazoan nuclei, with which it has been identified by Heidenhain (1894), R. Hertwig (1907), and others.

In the case of *Ascaris megalocephala*, as is well known, the ends of the chromosomes are cast out into the cell body in those blastomeres that are to give rise to somatic cells, this process being known as "chromatin diminution" (Boveri, 1899). This eliminated chromatin disintegrates in the cell body, and ceases to be chromatin, though it may play a part in the differentiation of the embryo. A somewhat similar condition is found in the fly *Miastor* (Kahle, 1908; Hegner, 1912, 1914).

Complete dissolution of the nucleus and chromatin takes place in certain atypical or pathological conditions. For example, in the genesis of the "worm-shaped" or apyrene spermatozoa of prosobranch gasteropods, the nucleus breaks up into chromatic masses and these gradually dissolve and disappear in the cytoplasm. This is followed, not by the death of the cell, but by a remarkable differentiation of the cell into the "worm-shaped" spermatozoön. Somewhat similar dissolution of the nucleus, or karyolysis, takes place in many experimental or pathological conditions which are ultimately followed by the death of the cell.

Finally the nucleolus, when it is large, as it is in the germinal vesicle of the egg, may be cast out bodily into the cytoplasm there to undergo dissolution, and in all cases of mitosis it dissolves either in the nucleus or in the cytoplasm, and its substance ultimately mingles with the cytoplasm. In addition to all these chromatic substances, which escape into the cytoplasm during mitosis, much of the linin is also discharged into the cell body, such as the spindle, interzonal filaments, and indeed all portions of the linin except that within and sheathing the chromosomes.

c) DIFFERENTIATION PRODUCTS

Many experiments indicate that the life and activity of the cell is associated with the interchange between nucleus and cytoplasm. Processes of oxidation, assimilation, and regulation are known to be dependent upon this interchange, and the same is certainly true of differentiation. One of the simplest cases of differentiation is found in the formation of secretion products within cells, such as yolk, oil, and zymogen granules. These usually appear as minute granules or droplets, which then grow in size until they more or less completely fill the cell. Whether they are products of destructive or of constructive metabolism is not altogether clear; probably in some cases they are the former, in others the latter.

Yolk and zymogen begin to form in the vicinity of the nucleus and in many cases out of a granular mass which is either chromidia, mitochondria, or the granular substance surrounding the centrosomes and known as "sphere-substance." In some cases, perhaps in all, the granular body known as a "yolk nucleus" is composed chiefly of sphere substance derived from the interaction of nucleus and cytoplasm; according to the Hertwig school zymogen granules are always derived from chromidia, and pigment may come from the same source.

Intracellular fibrils, such as skeletal, muscle, and nerve fibrillae, are also derived from chromidia, according to Goldschmidt (1909, 1910), and hence are formed in large part by substances derived from the nucleus. On the other hand, Meves (1910) and Duesberg (1909) maintain that such intracellular differentiations are derived from mitochondria which are purely cytoplasmic in origin. The more probable view is that mitochondria are formed by the interaction of nucleus and cytoplasm, and that all other cellular differentiations are formed in the same way; and if all these cytoplasmic differentiations are produced by the action of chromatin on cytoplasm the chromatin is only one factor in their origin.

As a result of all these observations it is impossible to avoid the conclusion that the nucleus is intimately concerned in differentiations, and the mechanism of the "nuclear control" of the cell is at least suggested by the escape of chromatin and other nuclear substances into the cytoplasm and the formation there of various differentiation products such as mitochondria, sphere substance, fibers, granules, etc.

7. *Size relations of nucleus and cell body:*

In general, nuclei are relatively largest in cells which are least differentiated, such as embryonic cells, and in the early stages of oögenesis and spermatogenesis. In many such cases the nucleus nearly fills the entire cell, leaving only a thin layer of cytoplasm at the periphery. As the metaplasm and differentiation products in the cytoplasm increase, the nuclear volume decreases, relatively if not absolutely. For example, the ratio of nuclear volume to cell volume is about 1:1.3 in the early oöcytes and spermatocytes of the gasteropod, *Crepidula plana* (Conklin, 1912*a*), whereas in the egg just before maturation, the ratio is 1:3 if the yolk is omitted, but including all the yolk the ratio is 1:53.

In cleavage cells the volume of the nucleus is proportional to the volume of the undifferentiated cytoplasm in which it lies, and in general one can estimate the volume of the undifferentiated cytoplasm of a cell by the volume of its nucleus. In muscle cells in which the contractile substance occupies most of the cell body, the nuclei are small and densely chromatic, whereas they are large and contain much achromatin where the contractile substance occupies only a small part of the cell body as in the tail muscles of ascidian tadpoles. Eycleshymer (1904) found that the volume of the cell body in the striated muscle cells of *Necturus* increased about ten times as much as the nuclear volume during the development from the 8-millimeter embryo to the adult condition, owing to the great increase of the contractile substance. In nerve cells generally the nucleus is large and contains much achromatin; presumably the differentiation products occupy a relatively small part of the cell body, leaving a large proportion of fluid cytoplasm in such cells.

By subjecting egg cells of *Crepidula* to strong centrifugal force it is possible to throw most of the yolk into one-half of a dividing cell and most of the cytoplasm into the other half (Conklin, 1912*a*). In such cases the daughter-nucleus in the cell which contains most of the yolk remains very small, while the nucleus in the cell containing most of the cytoplasm becomes very large. If this centrifuging occurs during the maturation divisions, the polar bodies, which are normally the smallest cells ever formed from the egg, may become very large, and their nuclei, which normally are only chromosomes of the late anaphase, may also become large.

During fertilization stages, the cytoplasm is more abundant at the animal pole of the egg and the yolk at the vegetative pole; the spermatozöon usually enters the egg near the vegetative pole and consequently it remains small since it lies in a region rich in yolk, but poor in cytoplasm. On the other hand, the egg nucleus normally lies near the animal pole in an area rich in cytoplasm, but poor in yolk; consequently it is much larger than the sperm nucleus; but as the sperm nucleus approaches the egg nucleus it moves into an area rich in cytoplasm, and consequently when it reaches the egg nucleus it may be almost, if not quite, as large as the latter. If now the egg is centrifuged during fertilization stages so as to reverse the normal positions of yolk and cytoplasm, the egg nucleus remains small and the sperm nucleus becomes very large, thus again showing that the growth of the nucleus is dependent upon the volume of cytoplasm which surrounds it (Fig. 4 *B*).

Entirely similar conditions occur during the cleavage of the egg. Normally cleavage cells which contain much cytoplasm have large nuclei; those which contain little cytoplasm have small nuclei. In *Crepidula*, the first two cleavages divide the egg into cells of approximately equal size, each containing about the same amount of cytoplasm and yolk, and accordingly the nuclei in these cells are approximately equal in size. However, if the eggs are centrifuged during the first or second cleavages most of the yolk may be thrown into one half of the daughter-cells and most of the cytoplasm into the other; in such cases, the nuclei in the yolk-rich cells remain very small, while those in the cells rich in cytoplasm become very large (Fig. 4 *C, D*).

In considering the size relations of nucleus and cell body, it is necessary to remember that this also depends upon the stage in the division cycle of the cell. The nucleus is smallest in the anaphase of mitosis when it consists only of the condensed daughter-chromosomes. It is largest in the prophase just before the nuclear membrane disappears. During the interval between these two phases, the nucleus continues to increase in size and, in general, the longer this interval between mitoses, the larger the nucleus becomes. The great size of the nucleus in certain cleavage cells, which divide but rarely, is evidently correlated with the length of the "resting" stage. Also the great size of the nucleus

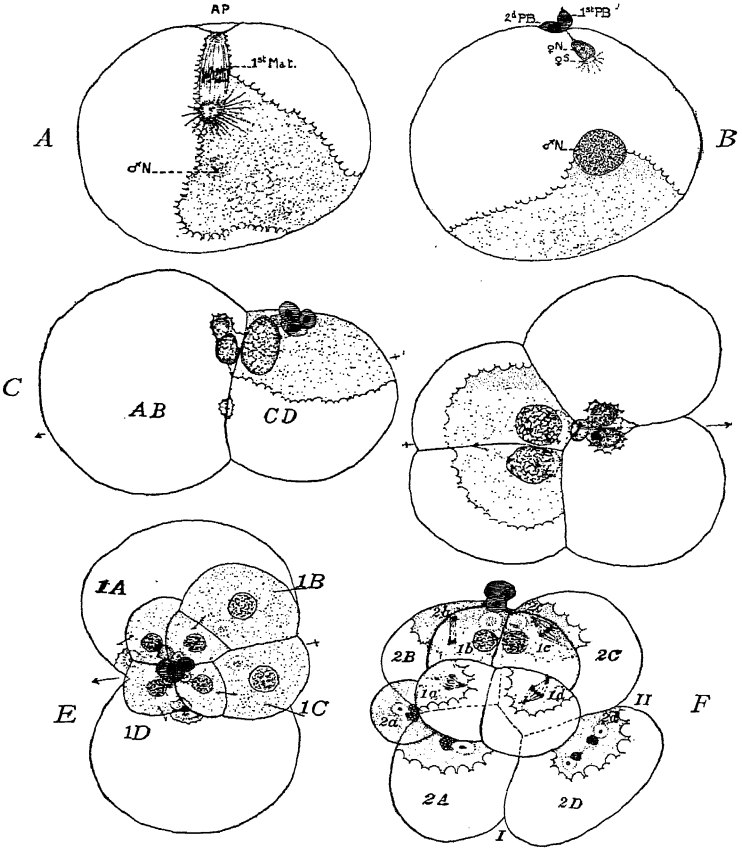

FIG. 4.—Eggs of *Crepidula* centrifuged in the 1- or 2-cell stages. *A*, Cytoplasm centrifuged away from the animal pole (AP) leaving first maturation spindle attached to that pole. *B*, Cytoplasm centrifuged away from animal pole leaving egg nucleus (♀N) and egg sphere (♀S) in an area of yolk, and sperm nucleus (♂N) in an area of cytoplasm. The latter is accordingly much larger than the former. *C*, Egg centrifuged during first cleavage, most of the yolk going into the cell AB, and of the cytoplasm into CD; daughter nuclei are proportional in size to the field of cytoplasm in which they lie. *D*, Four-cell stage of egg centrifuged during first cleavage; nuclei proportional to volume of cytoplasm in which they lie. *E*, Centrifuged during second cleavage separating purely protoplasmic macromeres (1B, 1C) from yolk-containing ones (1A, 1B). The four unequal macromeres have produced four micromeres of approximately equal size. *F*, Egg centrifuged at close of first cleavage so as to cause the second cleavage to be equatorial in position; first group of micromeres (1*a*–1*d*) not at animal pole, second quartet (2*a*–2*d*) forming in abnormal positions; evident attempt of cytoplasm to return as near as possible to animal pole.

of the egg before maturation and of the nuclei of many nerve cells is dependent in part upon the length of time since the last previous mitosis, during which time the nucleus continues to grow.

Finally, where there is an abnormal and unequal distribution of chromosomes to the daughter-cells, the cell which receives a small number will have a smaller nucleus than the cell receiving a large number. Boveri (1902, 1904, 1905) gives this as the only cause of inequality in daughter-nuclei, but it is plain that in normal development it plays a smaller part than the other two factors, namely, the volume of fluid cytoplasm in the cell and the duration of the growth of the nucleus, since the number of chromosomes usually remains the same in all cells of an embryo.

R. Hertwig (1903, 1908) has maintained that there is a definite ratio between the size of the nucleus and the size of the cell body; this he calls the "kern-plasma relation," or simply *k/p*. When this ratio rises beyond a certain figure, the cell divides, and the ratio is once more restored. However, in different blastomeres and tissue cells of the same species, this ratio differs enormously, depending upon the volume of fluid cytoplasm in the cell and the length of the resting period. There is no constant *k/p* for all cells of a given species, and departures from a given ratio are not the cause but rather a result of the rate of cell division (Conklin, 1912*a*). Not only the nucleus but also the centrosome and aster are proportional in size, other things being equal, to the volume of the undifferentiated cytoplasm. Also the volume of the chromosomes is proportional to the volume of the nucleus, and therefore ultimately to the volume of the cytoplasm.

These conclusions are of much importance in the study of differentiation, for one of the earliest and simplest forms of differentiation is that in which cells differ in size and rate of growth and division. If these differences could be shown to be due to differences in the size of chromosomes or nuclei, it would go far to establish the view of the nuclear control of differentiation. Following Boveri (1904, 1905), a great many investigators have found that where nuclei contain a larger number of chromosomes than is typical, the nuclei are larger than usual; where there is a smaller number of chromosomes, the nuclei are smaller; also the size of cells is sometimes larger when the nuclei are large and smaller when they are small. Consequently, it has been held that not only the size of the nucleus, but also the size of the cell was dependent upon the number of chromosomes. Without attempting to review in detail all of these investigations, it may be said that the relative number of chromosomes in a cell can have nothing to do with normal processes of differentiation of cells as to size and rate of growth and division, since in the same species every cell has typically the same number of chromosomes, and yet different cells come to differ enormously not only in size but also in form and content. Other things being equal, nuclei that contain less than the typical number of chromosomes are smaller, and those that contain more than the typical number are larger than those nuclei that contain the typical number of chromosomes. But where all nuclei contain the typical number, as is usually true in normal development, this cannot be the cause of size differentiations in cells. The real cause of such differentiations will be dealt with later. Incidentally, it may be said that differences in the size of nuclei or of homologous chromosomes in the same animal or plant are of no hereditary or differential value, as may easily be seen by considering the fact that the sperm nucleus, which is probably the smallest of all nuclei, has the same hereditary value as the egg nucleus, which in the stage before maturation is the largest of all nuclei.

III. DIFFERENTIATIONS OF DEVELOPMENT

A definite sequence of morphological differentiations, such as occurs in ontogeny, must be preceded at every stage by a definite organization (that is, differentiation and integration) of the developing substance, and in the last analysis by a definite organization of molecules and atoms. Many, and indeed most, morphological differentiations arise by a process of epigenesis or "creative synthesis," from other antecedent differentiations of a more fundamental and elementary nature. The great problem of development is to trace differentiations step by step to their earliest recognizable sources and to determine their intrinsic and extrinsic causes. Owing to the relatively large size of eggs and blastomeres and to the ease with which they may be subjected to experiment, they have for a long time furnished the principal material for the study of this problem. In tracing differentiations to their sources it is difficult if not impossible to reach the actual beginnings of any of them. At certain stages of development a differentiation becomes visible, or may be recognized by physiological or developmental processes, but its actual origin is indefinitely remote. The basis for the differentiation of cells is usually laid long before any differentiations can be seen with the microscope. Here, as in genetics, we recognize invisible differentiations not directly, but by means of development, which serves as an "indicator."

Logic alone led students of heredity to the conclusion that the fertilized egg must contain in some form or other antecedent differentiations out of which inherited characters develop. Weismann (1892) said that he tried for a long time to construct a purely epigenetic theory of heredity, but found this impossible, and therefore he was led step by step to what was at the time considered an extravagantly complicated theory of heredity. Recent experimental work on genetics and cytology has confirmed in the main Weismann's theory, and has added complexities of which he never dreamed. These earliest differentiations of the germ cells from which ontogeny proceeds are found chiefly in chromosomes and genes. But, although chromosomes and genes differ from one another, they undergo few if any differentiations in the course of ontogeny. One of the most striking and important discoveries in the cellular history of development is that the chromosomes and presumably the genes, even in the most highly differentiated cells, remain as they were in the fertilized egg. There is no evidence for the view, once advocated by Roux and Weismann, that the differentiation of cells is brought about by a qualitatively dissimilar distribution of chromosomes to those cells. On the contrary, there is abundant evidence that chromosomes are usually distributed equally and non-differentially to all somatic cells. Differentiations of the nucleus in different tissue cells, as for instance muscle and nerve cells, are generally limited to changes in shape and size, which are dependent upon differentiations of the cell body; they are effects rather than causes of histological differentiation.

While, therefore, we must look to the chromosomes as one of the most important factors in differentiation, it is in the cell body that these differentiations become manifest. Whatever the mechanism may be by which the invisible genes influence visible differentiations, it is certain that the progressive differentiations of development occur largely if not entirely in the cell body. Accordingly, we shall limit our attention to the differentiations of the cell body, and especially to those aspects of cytoplasmic differentiation which are concerned in early embryonic development. It is obviously impossible in this chapter to deal at all with the differentiations of organs or at all adequately with the histological differentiations of the multitudes of somatic cells of higher animals. We turn at once to the differentiations of the germ cells and of the early stages of ontogeny.

A. The Spermatozoön

The mature spermatozoön is one of the most highly differentiated cells of the metazoan body. Practically all of its visible peculiarities arise in the spermatid after the last maturation division. The spermatid is transformed into a spermatozoön by notable changes in shape and structure without intervening cell division. In this striking differentiation of a single, more or less isolated cell, every visible part of the spermatid undergoes change. In the formation of a flagellate spermatozoön the spherical nucleus contracts, forcing out achromatin and becoming small, elongated, and densely chromatic. The "ball centrosome" sends out the long axial fiber, which is the initial step in the elongation of the entire cell, nucleus as well as cytoplasm, while a "ring centrosome" is present in many cases, which grows out into the marginal filament of the tail. The cell body loses fluid, becomes immensely elongated, and may give rise to many differentiations such as perforatorium, middle piece, tail, and fin. These differentiations serve to bring the spermatozoön into the egg, and thereafter they disappear, either by being left outside the egg or by undergoing dedifferentiation within it. They take no direct part, therefore, in the differentiation of the embryo.

A striking instance of the extent to which unicellular differentiation may proceed is found in the apyrene or "worm-shaped" spermatozoa of prosobranch gasteropods. In this case the maturation divisions are omitted, and the single centrosome of the large first spermatocyte breaks up into a ring of granules which move to one pole of the cell. Here each granule sends out a filament which grows through the cell to the opposite pole. These filaments are parallel with one another, and as they continue to grow in length they stretch the cell out into the elongated and complex structure known as the "worm-shaped" spermatozoön. (See Meves, 1903; Kuschakewitsch, 1913; Reinke, 1914.) This peculiar spermatozoön does not fertilize the egg, and its complicated structures take no part in the differentiations of the embryo.

B. The Ovum

The differentiations of the egg are not so striking as those of the spermatozoön but they are much more persistent, and some of them give rise to important differentiations of development. Among these persistent differentiations are polarity, symmetry, and the localization of specific oöplasmic substances in definite regions of the egg.

1. *Polarity:*

The polarity of the egg is the earliest recognizable and most fundamental differentiation of morphogenesis; it is the chief factor in determining localization of developmental processes, such as the segregation of different oöplasmic substances and of specific physiological activities, the orientation of mitotic figures and cleavage planes, and finally the determination of the polarity and symmetry of the adult. In short, the polarity of the organism in the one-celled stage of development is the chief condition and cause of the polarity of all later stages.

Long ago Remak (1855) showed that the pigmented hemisphere of the frog's egg gave rise to the cells of von Baer's "animal germ layer," while the unpigmented hemisphere gave rise to the "vegetative germ layer." It is a notable fact that in all animals, with a few possible exceptions which are not well established, the ectoderm comes from the half of the egg lying nearest the animal pole, while the endoderm and the mesoderm come from the opposite hemisphere. It is important, therefore, to trace this earliest and most fundamental differentiation of morphogenesis to its source.

The polarity of the egg is indicated before maturation by a slight eccentricity of the germinal vesicle toward one pole, and usually the cytoplasm is more abundant at this pole while the yolk is more abundant at the opposite pole. In ovarian eggs the animal pole is usually the free pole while the egg is attached by its vegetative pole. However, Boveri (1901) found these conditions reversed in *Strongylocentrotus*, where the micropyle and the animal pole coincide with the attached pole of the ovarian egg. Jenkinson (1911), on the other hand, maintained that the micropyle and the animal pole of this egg lay at the free pole, but more recent work by Schaxel (1915) confirms Boveri's account.

If the chief axis of the egg, as well as of any other cell, is the line passing through the centrosome and nucleus of the resting cell, as Heidenhain maintains, it may be traced back in the oögenesis, not only in the oöcytes and oögonia, but also to the primitive sex cells and ultimately to the blastomeres, unsegmented egg, and oöcyte of the previous generation. The polarity of the egg may therefore be a persistent differentiation, as is the nucleus or centrosome, which is passed on from generation to generation, though like the nucleus and centrosome it also may undergo cyclical changes.

In certain eggs polar differentiation is marked, not only by the eccentricity of nucleus and centrosome, but also by the stratification of egg substances at right angles to the chief axis, and this stratification arises during oögenesis. For example, the frog's egg, before its escape from the ovary, has black pigment over the animal hemisphere, while the vegetative hemisphere is unpigmented; however, this differentiation does not appear until the later stages of oögenesis. In other cases such stratification occurs only after maturation. Thus Boveri (1901) found that the egg of *Strongylocentrotus* before maturation is covered all over by a uniformly distributed orange pigment. Immediately after maturation this pigment moves away from the animal and vegetative poles, and forms a denser zone of pigment just below the equator of the egg. In the eggs of *Dentalium*, Wilson (1904) found a condition similar to that in *Strongylocentrotus*, in which after maturation and fertilization there is a pigment-free cap at both poles of the egg and between these caps a reddish-brown zone.

In the eggs of *Myzostomum*, Driesch (1896) and later Carazzi (1904) found before maturation a dark-green mass at the vegetative pole and a uniformly distributed red pigment over the rest of the egg. Immediately after maturation and fertilization the red pigment assembles at the animal pole as a red cap, leaving a broad clear ring between this cap and the green mass at the vegetative pole.

In the eggs of *Styela (Cynthia) partita* (Conklin, 1905) an orange-colored pigment is uniformly distributed over the surface of the egg before maturation. Immediately after the entrance of the spermatozoön at the vegetative pole, this pigment with the superficial layer of cytoplasm flows to the vegetative pole where the orange pigment forms a cap, above which is a zone of clear cytoplasm, while the rest of the egg is filled with gray yolk (Fig. 5 *A–C*).

Many other cases have been described in which there is a marked stratification of the egg substances immediately after maturation or fertilization, although there may be no brightly colored pigments. Indeed, it is a general rule that such stratification occurs either before or just after maturation and fertilization.

2. *Causes of cell polarity:*

Any satisfactory explanation of the causes of polarity must be able to explain the following phenomena: (*a*) The typical localization of substances in cells, such as yolk at the vegetative pole and cytoplasm and nucleus at the animal pole, together with the typical orientation of spindles, centrospheres, and other cell constituents during and after mitosis. (*b*) The return of all cell

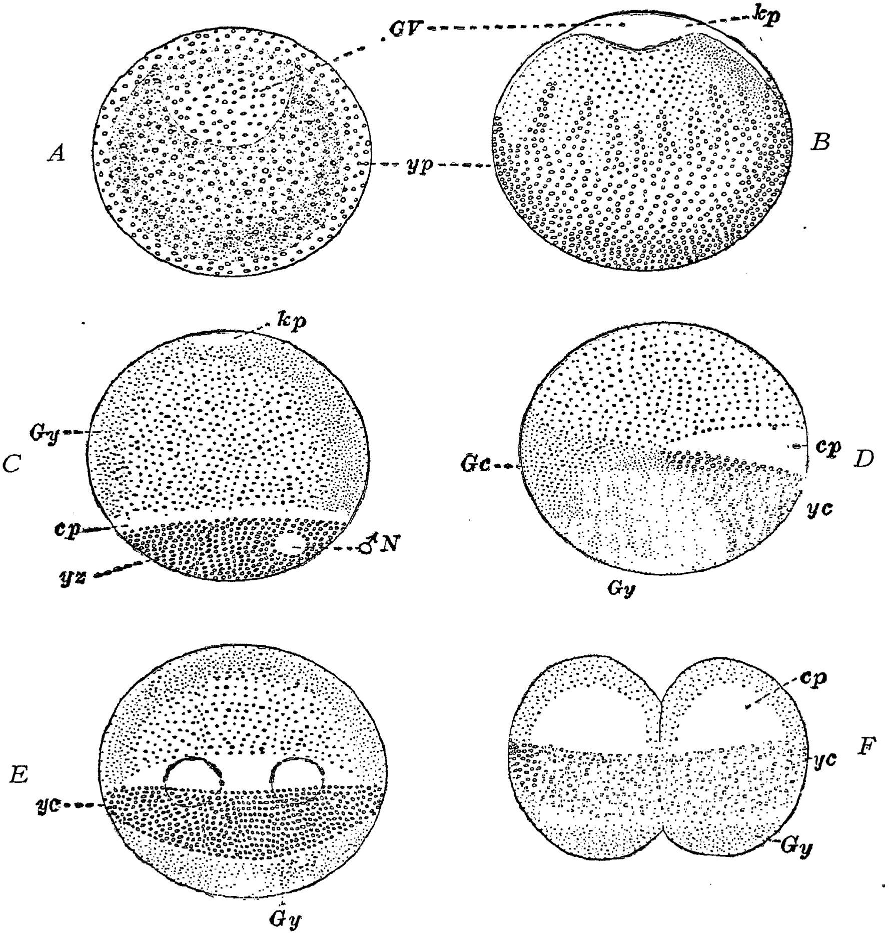

FIG. 5.—Surface views of living eggs of *Styela* (*Cynthia*) *partita*. Yellow spongioplasm (*yp*) containing mitochondria represented by small circles; gray yolk (*Gy*) by close stippling; clear protoplasm (*cp*) unshaded. *A*, Before fertilization, showing area of germinal vesicle (*GV*) and uniformly distributed yellow protoplasm (*yp*) over the surface. *B*, Immediately after entrance of sperm showing the clear karyoplasm (*kp*) from germinal vesicle at animal pole and streams of yellow protoplasm flowing to vegetative pole. *C*, Yellow protoplasm forming a zone (*yz*) at vegetative pole within which is sperm nucleus (♂N); above this a zone of clear protoplasm (*cp*) and one of gray yolk (*Gy*) with a remnant of karyoplasm (*kp*) at the animal pole. *D*, Left side of egg just before first cleavage showing half of yellow crescent (*yc*) and of clear protoplasm (*cp*) at posterior pole and half of gray crescent (*Gc*) at anterior pole. Between yellow and gray crescents is the area of gray yolk (*Gy*). The yellow crescent gives rise to mesoderm, the gray crescent to neural plate and chorda, gray yolk to endoderm, and the other substances to ectoderm. *E*, Egg similar to preceding, viewed from posterior pole. *F*, Two-cell stage viewed from posterior pole.

substances to their typical positions after they have been displaced, if time and opportunity for this return are given.

It is evident that the cause of polarity in cells is one of the most fundamental problems in the study of differentiation and regulation. The localization of formative substances is one of the chief factors in determining the localization of the parts of the developing embryo; and the return of these substances to their typical positions, when once they have been displaced, is a notable case of regulation in which the organization concerned is merely the polarity of a single cell. Because of the apparent simplicity of this problem of the polarity of the egg, the hope is raised that a thorough analysis of it may throw light on the problems of differentiation and regulation in general. In searching for the causes of polarity, one may deal with proximate causes, or more distant ones. Many of the hypotheses which have been proposed seek to trace polarity to its ultimate source, others attempt only to find the causes of polarity as they now exist in free or ovarian eggs. We shall take up first a consideration of these proximate, present causes, and later the more distant or ultimate causes of polarity.

It was formerly supposed that the polarity of the mature egg and even that of the cleavage stages was wholly dependent upon environmental conditions. Pflüger (1884) held that all axes of the frog's egg were alike in their developmental potencies; this condition he called the "isotropy" of the egg. Garbowski (1904) affirmed that in *Asterias glacialis* polarity is not determined even in the 8-cell and 16-cell stages and that the blastomeres are equipotential up to the 500-cell stage! On the contrary, Boveri (1901) showed that there was a marked stratification of the substances of the egg of *Strongylocentrotus* immediately after maturation and that even the ovarian egg shows polar differentiation. The results of much recent study of this problem make it seem extremely probable that the eggs of practically all Metazoa show polar differentiation at the time of, or even before, maturation.

a) GRAVITY NOT THE CAUSE OF POLAR DIFFERENTIATION OF THE EGG

The opinion has been frequently expressed that the polarity of the egg is determined by gravity. In the eggs of some animals, yolk has a greater specific weight than the plasma, and this fact led O. Hertwig (1893) to assert as a general law that "polar differentiation consists in this, that the lighter protoplasm collects at one pole and the heavier yolk substance at the other." Rhumbler (1899) also says, "Incontestibly the yolk granules (in telolecithal eggs) are collected in the lower part of the egg through their greater specific weights." This is true where yolk is much heavier than protoplasm or where eggs are subjected to centrifugal force of from several hundred to several thousand times the force of gravity, but there is no evidence that the organic polarity of the egg is determined by this factor, and there is the best of evidence that it is not so determined. Even in the frog's egg where the free egg floats with the yolk pole down and where, if the egg is inverted, the yolk will go through the egg to the opposite pole, it is certain that gravity does not determine polarity since the black and the white hemispheres which are plainly marked in the ovarian egg lie in the ovary in all possible directions with respect to the direction of gravity. In many accurately investigated cases among many different phyla of the animal kingdom the polarity of the egg is established irrespective of the direction of gravity; indeed, there are no well-authenticated cases in which the polarity of the ovarian egg is determined by gravity, and experiments on free eggs and embryos have demonstrated the "non-effect of gravity" in determining polar differentiation (Roux, Morgan, *et al.*).

Many attempts have been made to change the polarity of egg cells by means of gravity or centrifugal force, and it is a remarkable fact that the relative positions of almost all the visible substances in the egg may be changed without permanently changing the polarity. By means of centrifugal force, most of the yolk may be thrown to the animal pole and most of the cytoplasm together with the nucleus and centrosome to the vegetative pole, and yet if sufficient time is allowed all the parts will return to their normal positions, and later development shows that the polarity has not been changed.

b) NEITHER MATURATION NOR FERTILIZATION CAUSES POLARITY OF THE EGG

The formation of polar bodies usually takes place at the animal pole of the egg while the entrance of the sperm commonly occurs at the opposite pole. But this is not invariably true, and the exceptions prove that the real polarity of the egg is independent of the place at which the polar bodies form or the sperm enters. Häcker (1899) found in *Cyclops* that the fertilization pole was independent of the maturation pole and that both were independent of the position of the first cleavage spindle, and he therefore concluded, probably erroneously, that the direction of the first cleavage (presumably also the polarity of the embryo) is not preformed in the egg. In *Crepidula* (Conklin, 1917), under normal conditions the polar bodies invariably form at the animal pole, but if the eggs are centrifuged before the maturation spindles become attached to the peripheral layer of cytoplasm, the polar bodies may be caused to form at any place on the surface of the egg. As soon, however, as the egg is removed from the centrifuge, all its substances come back to their original positions, and development proceeds normally, although the polar bodies may lie at the vegetative pole or at any other place on the egg. This proves that the polarity of the egg and embryo is not the result of the formation of polar bodies at a particular point, but rather that the polarity of the egg antedates the maturation and under normal conditions is the cause of the location of the maturation spindles and polar bodies at the animal pole. Likewise, the place of entrance of the spermatozoön does not determine or modify the polarity of the egg or embryo.

c) SPONGIOPLASM OR GROUND SUBSTANCE

Many things indicate that the polarity of the egg and the orientation and localization of its constituent parts is due to a peripheral layer of ectoplasm and a central framework of viscid cytoplasm, and that the return of displaced parts to normal positions is due in the main to the persistent polarity of the ectoplasmic layer and to the elasticity or contractility of the framework. The peripheral layer surrounds the entire egg but is thickest at the animal pole. There is, therefore, a polar differentiation of this ectoplasmic layer, which is not changed by pressure or centrifugal force and which persists throughout development.

In eggs containing a considerable quantity of yolk, as in the eggs of *Crepidula* and *Styela* (Figs. 3 and 6), strands of the cytoplasmic framework or spongioplasm lying between the yolk spherules may be seen radiating from the animal pole, where they are most abundant, to the opposite pole, where they are least evident. Such strands connect the nucleus with the centrosomes, or centrospheres, of the resting stages, and the latter with the ectoplasmic layer. In dividing cells they are seen most plainly in the mitotic spindles and astral radiations.

Other contents of the egg consist of yolk, oil, water, pigment, and other inclusions and of a more fluid plasma, all of which lie in the meshes of this viscid framework. In *Crepidula* this viscid spongioplasm is very small in quantity compared with the other contents of the cell, in *Styela* it is relatively more abundant, and in other species it may constitute a still larger or an even smaller part of the cell contents.

By centrifugal force all the cell inclusions and the fluid plasma may be moved through this framework to any part of the egg, the yolk going to the heavy (distal) and the oil and fluid to the lighter (central) pole. At the same time the strands of the framework may be stretched or bent, but unless the centrifuging is strong enough to kill the egg, this substance is not stratified with the other cell contents. It is a notable fact that in dead, but not coagulated, eggs all the cell contents, including the spongioplasm, are more completely stratified by centrifugal force than is the case in living eggs.

In early stages of mitosis the amphiaster may be displaced with the more fluid contents of the cell, but after the metaphase the astral radiations around the centrosome become anchored to the ectoplasmic layer; thereafter the spindle may be stretched or distorted but it remains attached to the cortical layer even though the fluid cytoplasm surrounding the spindle may be driven away and its place taken by densely packed yolk (Figs. 3 *B*, 4 *A*). This proves that the spindle is not merely the expression of lines of force in a fluid medium, as Gallardo (1902, 1906, 1909), Hartog (1906), and Lillie (1908) supposed, but that it is a structure of a viscid or gelatinous character.

The same is true of the strands connecting the resting nucleus with the centrosphere and the latter with the peripheral layer. Experiments show that the centrosome, or rather the large centrosphere of the resting stages, is attached pretty firmly to the central pole of the nucleus, and however much the relative positions of different cell contents may be changed by pressure or centrifugal

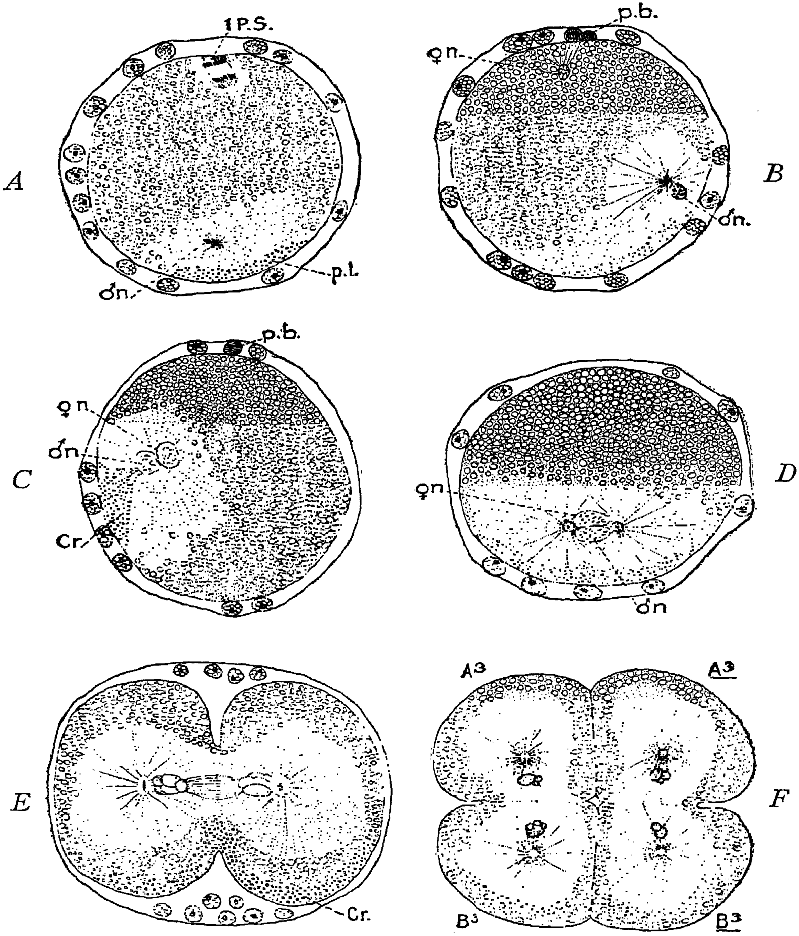

FIG. 6.—Sections of eggs of *Styela*, surrounded by chorion within which are follicle cells ("test cells") and polar bodies (*pb*). *A*, Showing first polar spindle (1 *PS*), sperm nucleus and aster (♂N) near vegetative pole, with substance of yellow zone (*pl*) at that pole. *B*, Sperm nucleus and aster (♂N) have moved to posterior pole where they wait for the egg nucleus (♀N), yellow zone being drawn to posterior pole. *C* and *D*, Meeting of sperm and egg nuclei and formation of first cleavage spindle and yellow crescent (*Cr*); *C* is cut in the polar axis and in the plane of bilateral symmetry; *D* is an equatorial section through the egg. *E*, Equatorial section in the telophase of the first cleavage, showing yellow crescent (*Cr*) at posterior pole. *F*, Equatorial section during telophase of second cleavage.

force, this attachment of nucleus to centrosphere and of the latter to the periphery is rarely broken, though it may be greatly stretched and distorted. Even though nucleus and centrosphere are forced from the animal to the vegetative pole, the centrosphere continues to lie between the nucleus and the animal pole, and visible strands of spongioplasm connect it on one side with the nucleus and on the other with the ectoplasm of the animal pole (Fig. 3 *C–F*). Thus it happens that the position of most of the egg contents may be changed without permanently altering its polarity, as is shown by the fact that these contents usually come back once more to their normal positions as soon as the pressure is removed. Also the position and direction of the mitotic figure and of the resulting cleavage plane may be changed experimentally without changing the polarity. In short, the original polarity of the egg persists in the organization of the ectoplasmic layer and the spongioplasmic framework after the positions of nuclei, centrospheres, mitotic figures, cleavage planes, and various inclusions such as yolk, oil, pigment, etc., have been changed. And so I conceive that polarity, and what is generally implied by the term "organization of the egg," resides in this cortical layer of ectoplasm and the internal framework of spongioplasm.

These conclusions may be harmonized with the observations of Lyon (1907), Morgan (1909, 1910), Lillie (1909), and others who have found that many of the visible substances in eggs may be moved in any direction without changing the polarity and organization of the egg and embryo; in particular they agree with and extend the conclusions of Lillie (1906, 1909) that this organization persists in the "ground substance" of the egg. However, in certain eggs this organizing, orienting ground substance may be relatively small in volume as compared with other contents of the egg.

The following hypotheses deal with initial or ultimate causes of polarity. Most of them are founded upon analogies or supposedly logical necessities, rather than upon direct observation of the visible polarity of egg cells.

d) AXIAL GRADIENT OF METABOLISM

In a long series of contributions and in several books, Child (1911, 1912, 1913, 1914, 1915, 1916, 1917, 1919, 1923) has maintained that morphological polarity is the result of physiological activity, or what he calls the "axial gradient of metabolism." He finds that oxidation is more rapid at the anterior end of an animal or embryo, or even of a fragment of an animal or embryo, than at the posterior end. Similar conditions are found at the animal pole of an egg as contrasted with the vegetative pole, and in apical portions of plants as contrasted with basal portions. Furthermore, Child finds that new polarities may be established experimentally in pieces of an organism whenever in these pieces a new physiological gradient is set up.

> The facts indicate that polarity and symmetry originate as physiological reactions of specific protoplasms to differentials of some sort in the environment of the protoplasm concerned. These reactions give rise to gradations in physiological condition,

> the physiological gradient, which provide a physiological basis for the realization of different hereditary potentialities in different regions, cells or cell groups [1923, p. 353].

Elsewhere he says:

> This viewpoint by no means excludes the possibility that a gradient, once established, may persist through reproductive processes and so be hereditary for a later individual or generation. Certainly in many such cases, very probably in all, inheritance is cytoplasmic, i.e., the gradient simply persists in the cell or cell mass as it does in many cases of organic reproduction. This may be the case also in some eggs, while in others the differential to which the growing oöcyte is exposed in the ovary may determine polarity, and conditions connected with fertilization, union of pronuclei, the first cleavage spindle, or some other factor may determine bilaterality [1923, p. 352].

Thus the particular causes of the polarity and symmetry of eggs are regarded as variable and rather indefinite; in some cases polarity may be inherited, in others it may be established by conditions in the ovary, but in all cases its *initial* cause is supposed to be the physiological gradient. But whether the increased metabolism at the animal pole of an egg, which Child has discovered, is the cause or the result of polar differentiation has not been determined with certainty, and in what material form this physiological gradient is initially embodied is not even suggested.

Bellamy (1919), working in Child's laboratory, announced the interesting discovery that the polar differentiation of the frog's egg is determined by its blood supply, the pole of the egg which receives arterial blood becoming the animal (black) pole while that from which the venous blood returns becomes the vegetative (white) pole of the egg. Later, however (1921), he corrected this and showed that there was no constant relation between the blood supply and the polarity of the advanced oöcyte, though he still considered that there might be such a causal relation in earlier stages of oögenesis. If the animal pole of the egg is determined by a greater supply of nutriment or oxygen than that which goes to the vegetative pole, one would expect that the attached pole of the oöcyte would always be the animal pole since it lies next to the blood supply, but with the single exception of the echinoderms this is not the case.

e) ELECTRIC POLARITY

The suggestion has been made that organic polarity may be of the nature of electric polarity, but there is little if any evidence that this is true and there are some strong evidences against it. Neither the entire egg nor any of its parts orients with respect to a constant current passed through the water in which eggs are placed, and none of the constituent parts of a cell that are visible microscopically are moved by an electric current passed through the cell itself, unless that current is strong enough to produce convection (Conklin, 1912). Furthermore, if organic polarity were the result of electric charges on colloidal particles or if it were due to different electric properties of the cell membrane at the two poles, the polarity of fused eggs should be the resultant of the polarities of the components, but this is not the case. When two or more eggs of *Crepidula* stick together so that they constitute a single mass, the original polarity of each component persists. These facts indicate that organic polarity is not the result of electrical polarity.

Hyman and Bellamy (1922) have found in developed animals of many phyla that there is a correlation between metabolic gradients and electrical gradients, regions of higher metabolic rate being externally negative and internally positive to regions of lower rate. But they conclude, probably correctly, that the electrical gradient is the result of the metabolic gradient rather than the reverse.

f) MOLECULAR POLARITY

Driesch has for a long time regarded the polarity and symmetry of an egg as the resultants of the polarity and symmetry of constituent particles, presumably molecules or molecular aggregates. Przibram (1906) in particular has maintained that there is essential similarity between the polarity of crystals and that of organisms. Undoubtedly there are many superficial resemblances between the two; in particular the capacity of broken crystals to regulate or regenerate strongly suggests the similar capacity of organisms. And yet it is probable that these resemblances are only analogies. Nevertheless, it may well be true that the ultimate causes of organic polarity are to be found in the polarity of molecules or colloidal aggregates of molecules. However, the immediate cause and expression of the polarity of the egg is to be found in the character and localization of the ground substance, or, more specificially, of the ectoplasm and spongioplasm of the egg.

3. *Symmetry:*

Eggs are usually spherical in form with one chief axis which is heteropolar and two cross-axes which are homopolar. Such an egg is radially symmetrical. A few types of eggs, especially those of insects and cephalopods, are not spherical but are elongated and have all three axes different, and the chief axis and one of the cross-axes heteropolar. This is the condition of bilateral symmetry, and even in the ovarian eggs of insects and cephalopods all the axes and poles of the future animal can be clearly recognized.

In cases where the egg is spherical and apparently radially symmetrical there may be indications that the intimate structure of the egg is bilateral. For example, in ascidians, Amphioxus, and the frog, bilaterality is plainly marked in the egg after fertilization and before the first cleavage. In all of these the animal pole is marked, before maturation and fertilization, by the eccentricity of the germinal vesicle and the greater accumulation of cytoplasm at that pole, and in the frog's egg the animal hemisphere is covered by black pigment. There is, however, no visible bilaterality of these eggs before fertilization, and yet under typical conditions bilaterality appears in such manner as to indicate that it is already prepared for in the internal structure of the egg.

For example, in the egg of the ascidian *Styela* (Conklin, 1905) the egg substances are apparently radially symmetrical before maturation and fertilization. There is a central mass of gray yolk, a large, transparent germinal vesicle near the animal pole, and a superficial layer of cytoplasm containing yellow or orange pigment and great numbers of minute spherules, which Duesberg (1915) identifies by their staining reactions as mitochondria (Fig. 5). In other species of ascidians this peripheral layer may be red, violet, or entirely lacking in color, but it always contains the mitochondria; for example, the living eggs of *Phallusia mamillata* are clear as glass, as Driesch (1895) has said, but this same mitochondrial layer can be plainly differentiated in this species by its staining reactions (Conklin, 1911). In *Styela* a small portion of this yellow substance is also found around the germinal vesicle, as well as later around the nuclei of the cleavage cells, and there seems to be good evidence that it is formed in the immediate vicinity of the nucleus and later migrates to the periphery of the egg. In its manner of origin and distribution it resembles sphere substance.

The wall of the germinal vesicle breaks down and the small first maturation spindle is formed near the animal pole and lies in the transparent material derived from the germinal vesicle, until after the spermatozoön enters the egg, when the first polar body is formed. The spermatozoön enters the egg near the vegetative pole, either exactly at that pole or within 30° of it. Immediately after its entrance the superficial layer of yellow cytoplasm, containing mitochondria, flows to the point of entrance where it forms a yellow cap; the clear substance derived in the main from the achromatin of the germinal vesicle also flows to the vegetative pole, and forms a clear zone just above the yellow cap. Up to this time all the substances of the egg are apparently radially symmetrical (Figs. 5 *A*, *B*, 6 *A*).

The sperm nucleus and aster then move away from the vegetative pole along a meridian of the egg, the sperm aster divides and forms the first cleavage spindle at right angles to the chief axis, and this spindle and the sperm nucleus come to rest near the equator of the egg and at a point which later development shows is posterior. Here they wait for the egg nucleus to come down from the animal pole after the formation of both polar bodies. As the sperm nucleus and aster move to this posterior pole they draw after them the material of the yellow cap and clear zone, and coincidently with the formation of the spindle the yellow substance takes the form of a crescent around the posterior side of the egg. At about the same time a light-gray crescent is formed around the anterior side of the egg. All the egg substances are now bilaterally symmetrical, the plane of symmetry passing through the middle of both crescents and also through the middle of the first cleavage spindle; consequently the first cleavage furrow divides the egg into equivalent right and left halves (Fig. 5 *F*).

Is this bilaterality determined by the path of the sperm nucleus, or is the latter determined by the former? The following facts indicate that the sperm moves in a pre-established path: (1) The sperm nucleus does not always take the shortest path to the equator; e.g., when it enters 30° to one side of the vegetative pole it sometimes moves across the chief axis and takes the longest path

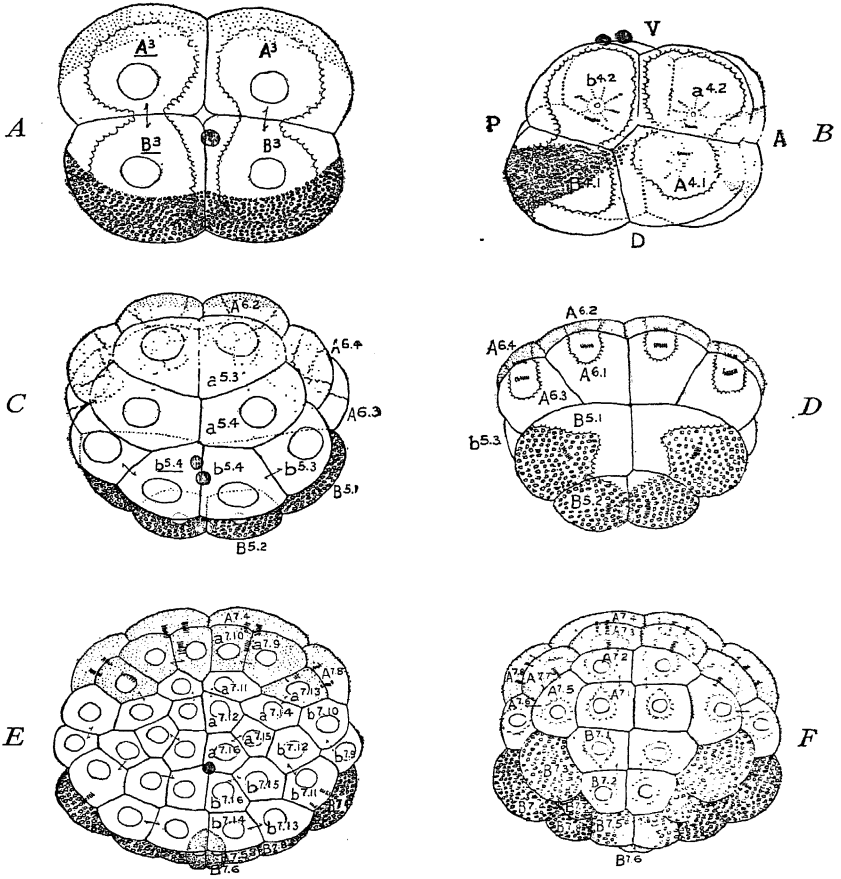

FIG. 7.—Entire eggs of *Styela; A, C, E,* viewed from the animal (ventral) pole; *B,* from right side; *D* and *G,* from vegetative (dorsal) pole. Yellow crescent shaded with small circles, chorda and neural plate with stipples. *A*, Four cells. *B*, Eight cells. *C* and *D*, Twenty cells. *E* and *F*, Sixty-four cells.

to the equator (Fig. 6 *A*, *B*). (2) It moves to the equator and there stops a distance from the surface and waits for the approach of the egg nucleus. (3) The first cleavage spindle always forms at this point parallel with the surface and at right angles to the chief axis (Fig. 6 *D*). These facts indicate that it is not the accidental point of entrance of the sperm nor the chance path of the sperm nucleus within the egg which determines the place at which egg and sperm nuclei meet, and consequently the posterior pole and the plane of bilateral symmetry. Nor will chance movements of any of the egg contents account for the location of the first cleavage spindle at right angles to the chief axis, near the posterior pole and near the equator of the egg. Rather the movements of the sperm nuclei and of the egg substances take place along pre-established lines. Although the bilaterality of the egg does not become apparent until after these nuclei and substances have moved out of the chief axis, the causes of these definite movements must be found in the structure of the egg itself (Conklin, 1905).

Entirely similar conditions are found in the egg of Amphioxus, according to Cerfontaine (1906). Here the sperm enters at the vegetative pole and moves up to or a little above the equator on the posterior side of the egg where it meets the egg nucleus and where the first cleavage spindle is formed. From this time all the poles and axes of the future embryo are clearly indicated. Cerfontaine holds that the bilaterality of the egg is indicated by the distribution of yolk in the egg before its fertilization and indeed in the oöcyte of the first order.

In the frog's egg, Roux (1887, 1895, 1903) found that the spermatozoön enters near the boundary between the white and black hemispheres, and immediately afterward the pigment layer of the black hemisphere is tilted over toward the side on which the sperm enters, thus exposing a crescentic area on the opposite side of the egg which is neither white nor black but gray in color (Fig. 9 *A*, *B*). Later development showed that this "gray crescent" is anterior in position, the point of entrance of the sperm is posterior, and the path of the sperm nucleus in the egg ("copulation path") lies in the plane of the first cleavage, which coincides with the median plane of bilateral symmetry in almost all cases. By means of localized fertilization, in which the spermatozoa were artificially applied to the egg at any point desired, Roux found that the point of entrance of the sperm still marked the posterior pole; and the copulation path coincided with the plane of the first cleavage and the median plane of the embryo just as in cases of normal fertilization, and he therefore concluded that the posterior pole and the plane of bilateral symmetry are determined by the point of entrance of the spermatozoön (Fig. 9 *B*).

Roux's results have been largely confirmed and extended by later investigators, particularly by Morgan and Tsuda (1894), Schultze (1900), Moskowski (1902), and Brachet (1904, 1910, 1911). The latter finds that there is a primary bilaterality of the egg, which may be altered by environmental influences such as gravity, the point of entrance of the sperm, dispermy (Herlant, 1911), etc.; however, if eggs are caused to develop parthenogenetically by Bataillon's method of *picûre*, this primary bilaterality is not altered. In short, the frog's egg, like those of the ascidian and Amphioxus, has a primary bilaterality

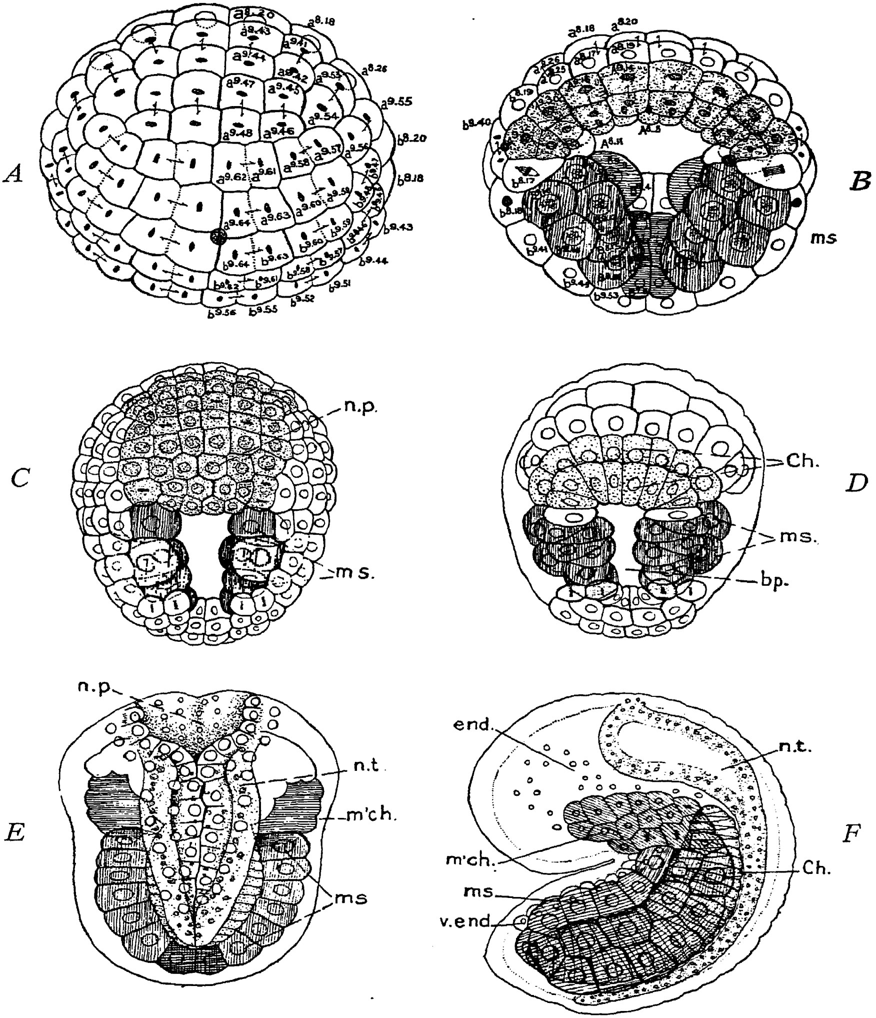

FIG. 8.—Later stages of *Styela;* neural plate and chorda shaded by stipples, muscles by vertical lines, mesenchyme by transverse lines. *A*, *B*, Ventral and dorsal views of 180-cell stage. *C*, *D*, Dorsal views of elongated gastrula. *E*, Early tadpole showing neural tube (*nt*); mesenchyme (*m'ch*); muscles (*ms*). *F*, Left side view of tadpole; gastral endoderm (*end*); nerve tube (*nt*); chorda (*Ch*); mesenchyme (*m'ch*); muscles (*ms*); ventral endoderm (*v. end*).

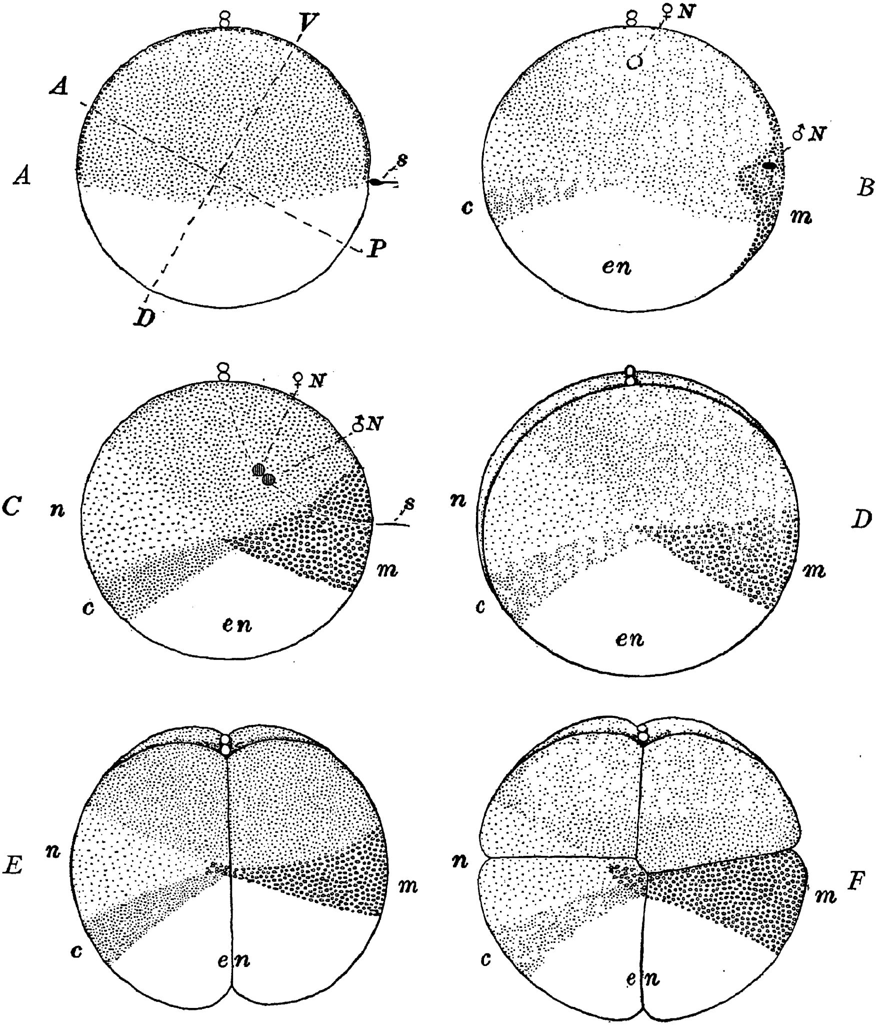

FIG. 9.—Schematic views of frog's eggs seen from right side; axes of future embryo shown in *A*; pigmented hemisphere stippled; hypothetical mesodermal crescent (*m*) around posterior side of egg; gray crescent containing substance for chorda (*c*) and neural plate (*n*) around anterior side; endoderm (*en*) comes from unpigmented portion at vegetative pole.

determined by the intimate structure of the egg itself, but this primary bilaterality and the localization of egg substances may be secondarily shifted by certain environmental influences.

Eycleshymer (1915) sums up the evidence regarding the origin of bilaterality in the vertebrate embryo as follows: "Neither the position nor direction of the cleavage grooves has the slightest significance as far as the setting apart of definite embryonic areas is concerned." He agrees with Whitman that bilaterality precedes cleavage. Smith (1922) finds in *Crytobranchus* that localized fertilization does not influence the plane of bilateral symmetry, but does tend to be at right angles to the plane of the first cleavage. There is no constant relation between the latter and the median plane of the gastrula, and bilateral symmetry does not appear until the late blastula stage. On the whole, the evidence is clear that in many vertebrates the egg is bilaterally symmetrical at the beginning of cleavage, but the cleavage furrows may bear no constant relation to this organization of the egg.

Another case, and probably the very first discovered in which a spherical egg has visible bilateral structure, is that of the gasteropod *Neritina fluviatilis*. Here Blochmann (1882) found, on the right and left of the polar bodies, a mass of coarse granules which he traced into the right and left velar cells and which he therefore called "Urvelargranula." So far as known this is the only indication of bilateral structure in the unsegmented egg of a gasteropod, and it leads one to suspect that in many other eggs there may be faint or obscure indications of bilaterality that have not been discovered. An intimate bilaterality of the egg has been described by Bartelmez (1912) in the pigeon and by various authors in fishes, nematodes, and rotifers.

Eggs and cleavage stages are radially symmetrical in all animals with radial symmetry. In ctenophores, which are biradial (disymmetrical), the cleavage is biradial. But in bilateral animals bilaterality may not become apparent until the late cleavage or the blastula or gastrula stage. Thus the eggs and early cleavages of echinoderms, polyclades, annelids, and mollusks, other than cephalopods, are visibly radial (or spiral) in symmetry; this gives place to visible bilaterality at different stages—at the 25-cell stage in *Crepidula*, the 32-cell stage in *Nereis*, the 44-cell stage in *Planocera*, and not until the gastrula stage in echinoderms. But in annelids and mollusks the spiral or radial symmetry of cleavage is only superficial, for experiments demonstrate that the blastomeres of the 2-cell and 4-cell stages are already differentiated for particular halves or quarters of the embryo. On the other hand, in echinoderms these blastomeres do have equal potency, and consequently it has been maintained that the eggs and cleavage stages of echinoderms are radially symmetrical. However, certain experiments show that in normal development the echinoderm egg is bilateral from the 2-cell stage on; Boveri (1907) found that in some cases the sperm nucleus might go entire into one of the first two cleavage cells, leaving the other cell with only its half of the egg nucleus ("partial fertilization").

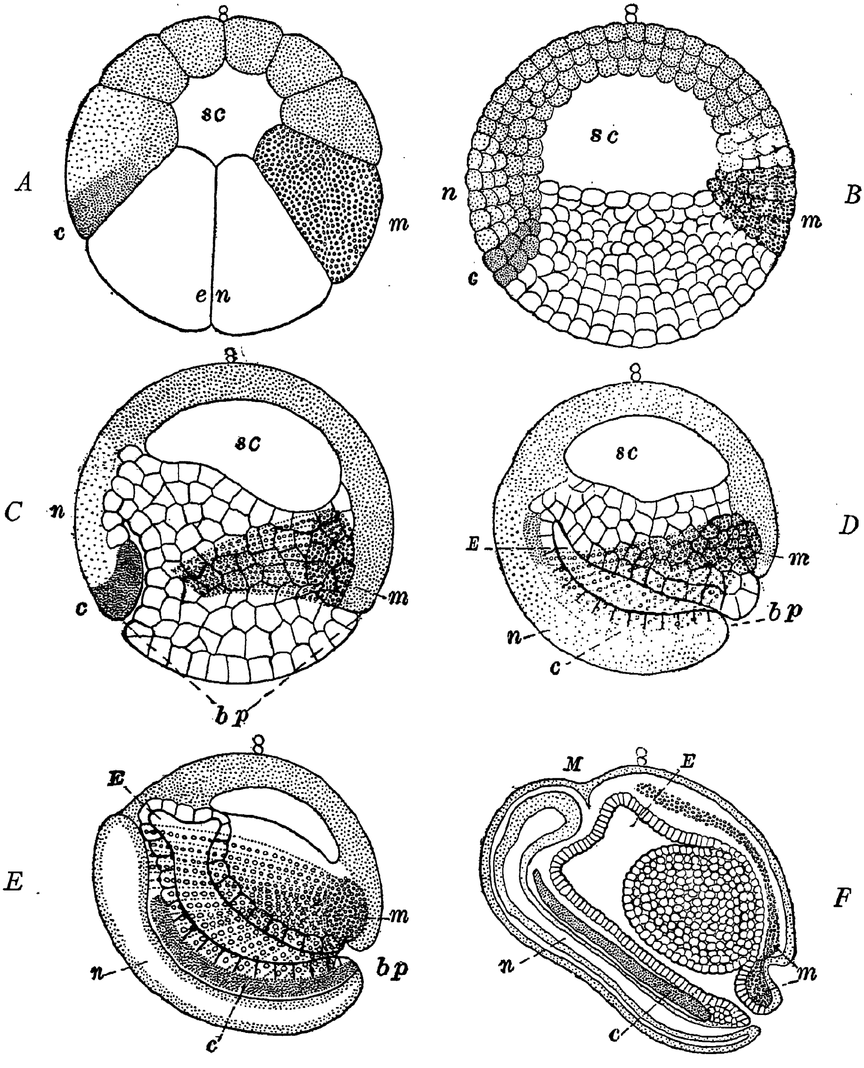

Fig. 10.—Median longitudinal sections of frog's eggs and embryos; axial relations and shading as in preceding figure.

In such eggs the diploid nuclei derived from the fertilized half are larger than the haploid nuclei of the other half; correspondingly the right or left half of the larva had larger nuclei than the other half. Consequently the first cleavage was in the plane of bilateral symmetry, and probably the echinoderm egg is bilateral under normal conditions.

Where all three axes and all of their poles differ we have asymmetry. Such a condition is found in the visceral mass of gasteropods, in the limbs of certain Crustacea, and in the internal viscera of many vertebrates. This asymmetry is almost always derived from bilateral symmetry and in the case of gasteropods it can be recognized in the gastrula and can be traced back through a series of alternations in the direction of cleavage to the very first division of the fertilized egg (Conklin, 1897). Crampton (1894) found that these alternations in the direction of cleavage in sinistral gasteropods, such as *Physa*, are the reverse of those in dextral forms such as *Lymnaea*. The direction of the first cleavage, whether dexiotropic or laetropic, is determined by the structure of the unsegmented egg and consequently the asymmetry of the adult gasteropod, whether dextral or sinistral, is determined before cleavage begins. It has not been found possible in gasteropods to change experimentally dextral to sinistral asymmetry or the reverse, but this has been done in the case of the asymmetrical limbs of certain Crustacea. Finally, it seems necessary to assume that the symmetry of the egg coincides with the symmetry of the developed organism; and that this polarity and symmetry of the egg exists not merely in the polarity and symmetry of constituent molecules or particles but also in the molar orientations of formative substances. I see no way of accounting for this primary symmetry of the egg except as I have attempted to explain polarity, namely by the peculiar structure of the ectoplasmic layer and the spongioplasmic framework. In these rather than in the positions of nuclei, centrospheres, yolk, oil, pigment, and other inclusions, symmetry inheres and persists.

4. *The egg pattern:*

In his pioneer work on developmental mechanics, *Unsere Körperform*, Wilhelm His (1874) propounded the doctrine that the organs and parts of an embryo arise in the early stages of development, perhaps even in the unsegmented egg, from definitely localized areas. "The principle according to which the germinal disk (of the chick) contains the preformed germs of organs spread out over a flat surface, and conversely, that every point of the germinal disk is found again in a later organ, I call the Principle of Organ-forming Germ-regions [*Organbildende Keimbezirke*]." This principle has been denied in its totality by certain authors, and certainly the form in which it was stated by His is open to some criticism.

This principle of His was based upon logic rather than upon direct observation and experiment, but today many cases are known in which certain organs can be traced back in ontogeny to certain cleavage cells, and even to plainly marked regions of the unsegmented egg. Before maturation and fertilization such regions are not usually recognizable, but at the beginning of cleavage the eggs of many animals are mapped out into visibly different areas which will, under typical conditions, give rise to specific parts.

This egg pattern differs in different phyla and classes. Coelenterata in general constitute a type in which the egg substances are concentric in arrangement, although showing polar differentiation in the chief axis. In the scyphomedusan, *Linerges mercurius* (Conklin, 1907), there is a peripheral layer of clear ectoplasm, which is thickest at the animal pole; beneath this is a layer rich in yolk, while the center of the egg is occupied by a more fluid mass containing large vacuoles. This central mass becomes in the main the semifluid contents of the blastocoel, while the other layers may be seen in the cells of the blastula and gastrula in the same relative positions as in the unsegmented egg. Although typically endoderm is formed from the oöplasm at the vegetative pole and ectoderm from that over the animal hemisphere, no differences can be seen in the oöplasm at these two poles in *Linerges*. Similar conditions are found in many other coelenterates. In Cnidaria these oöplasmic substances are radially symmetrical around the chief axis, whereas in ctenophores they are biradial as is shown by the early cleavages. In other phyla there is frequently found a more or less concentric arrangement of egg substances in the immature egg, but after maturation this arrangement gives place to a stratification at right angles to the chief axis.

Polyclads, annelids, and mollusks, with the exception of cephalopods, have an egg pattern which is much the same in all three. The ectoderm is formed from transparent cytoplasm in the region of the animal pole, and in many cases it can be seen that a large part of this transparent plasma comes out of the germinal vesicle at the time of the first maturation division. In living eggs of fresh-water pulmonates, *Lymnaea*, *Physa*, and *Planorbis*, this clear plasm may be traced from the germinal vesicle until it forms a clear cap at the animal pole, then spreads out over the upper hemisphere and finally enters into the ectoderm cells (Conklin, 1910). In these pulmonates the substance which goes to form the endoderm and mesoderm is rich in yolk and yellow in color; it occupies the vegetative hemisphere of the egg, and is gradually covered by the downgrowth of the layer of clear plasma (Fig. 11).

Essentially similar localizations of oöplasmic substances are found in other gasteropods, lamellibranchs, annelids, and polyclads. Furthermore, apical sense organs, cerebral ganglia, prototroch and velum, mouth and intestine, arise from corresponding regions of the egg in these three phyla, although the egg substances which enter into these organs are not clearly differentiated in the unsegmented egg. Different types of egg patterns are found in other phyla, though in general each phylum has its particular pattern. The pattern differs in rotifers, nematods, insects, cephalopods, and chordates and, in each of these, pecularities of pattern give rise to peculiarities of adult organization. In

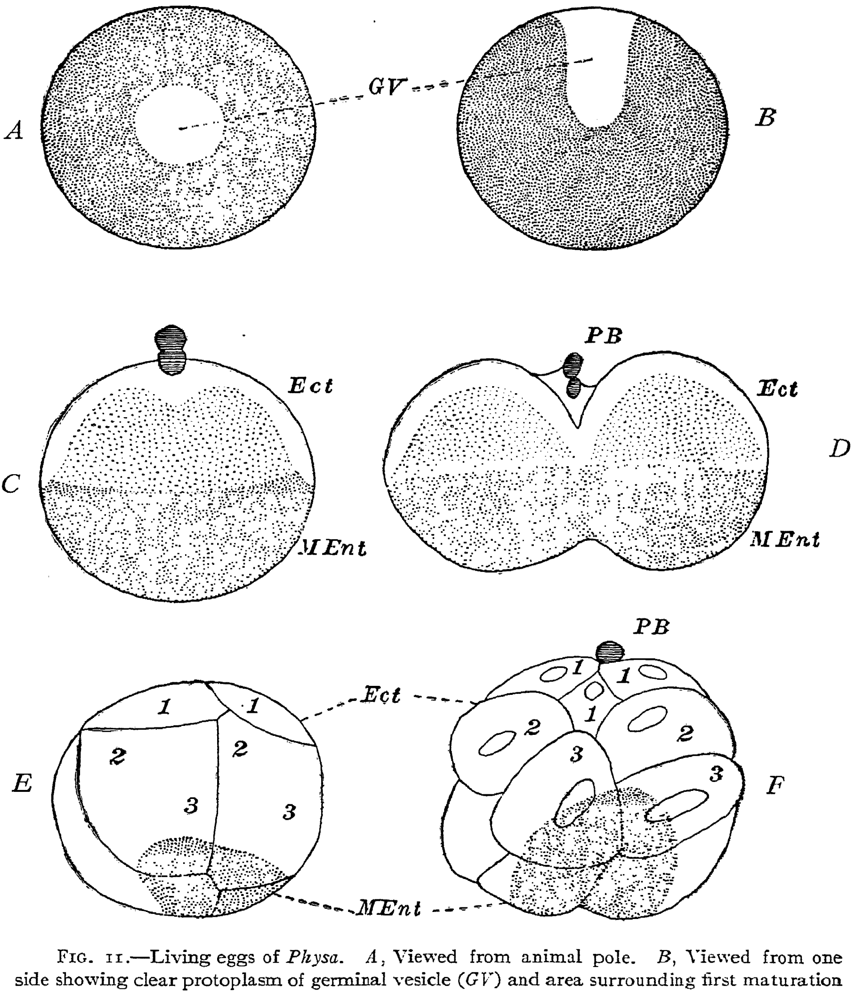

FIG. 11.—Living eggs of *Physa*. *A*, Viewed from animal pole. *B*, Viewed from one side showing clear protoplasm of germinal vesicle (*GV*) and area surrounding first maturation spindle; deeply stippled area is yellow yolk. *C*, Clear ectoplasm (*ect*) has extended down over the upper hemisphere leaving yellow mesentoplasm (*MEnt*) uncovered in lower hemisphere. *D*, Telophase of first cleavage, showing positions of ectoplasm and mesentoplasm. *E*, Eight-cell stage showing two of the first quartet cells *1*, and positions where second *2*, and third *3*, quartet cells will form. *F*, Twelve-cell stage just before formation of third *3*, quartet showing yellow mesentoplasm at vegetative pole (*MEnt*) uncovered by ectoplasm (*Ect*).

insects not only are all the axes and poles of the embryo marked out by the shape of the egg, but some of the future organs are indicated by the differentiated areas of cytoplasm. Thus the sex cells form at the posterior pole of the egg from an area of "posterior pole plasm," and peculiar coarse granules, "the germ cell determinants" (Hegner, 1914), located at this pole, go into the germ cells.

In the chordates the egg pattern differs from that of any of the invertebrates as fundamentally as the adult form does. It has often been noted that the vertebrate body is inverted as compared with that of many invertebrates, the nervous system being dorsal in one and ventral in the other; the same is true of the egg pattern. The animal pole of the egg and the center of the ectodermal area is nearly dorsal in mollusks, nearly ventral in ascidians, Amphioxus, and amphibians, and correspondingly the orientation of many organ-forming regions of the egg is inverted in vertebrates as compared with invertebrates.

In *Styela* the yellow crescent around the posterior border of the egg gives rise to all the mesoderm of the larva, the two horns of the crescent and its middle portion becoming mesenchyme, while the rest of the crescent goes into the muscle cells of the larval tail. The gray crescent around the anterior border goes into the neural plate and notochord, its dorsal portion giving rise to the former and its ventral portion to the latter. The slate-gray substance at the vegetative pole between the two crescents becomes endoderm, while the clear cytoplasm, which before the first cleavage lay just above (ventral to) the yellow crescent, spreads out over the animal hemisphere and becomes the ectoderm of the embryo (Figs. 5, 6, 7, 8). A similar localization of differentiated oöplasmic substances is found in Amphioxus and the frog. In the latter the gray crescent around the anterior side of the egg gives rise to neural plate and notochord, while there is good evidence that the mesoderm comes from a crescentic area around the posterior border of the egg; endoderm arises from the non-pigmented area at the vegetative pole and ectoderm from most of the pigmented area at the animal pole.

5. *"Organ-Forming Substances":*

It does not follow, however, because specific substances are located in particular regions of the egg and typically enter into particular organs that they are, therefore, "organ-forming substances" (Rabl, 1906). Many of these substances such as yolk, oil, and pigment are mere inclusions in the cytoplasm, and are passively located in particular regions or are passively moved from one place to another. Often it is possible to displace these substances by centrifugal force and get normal development. This has been done by Lyon (1907) in the case of echinoderms, Morgan (1909, 1910) in the case of echinoderms and lamellibranchs, Lillie (1909) in annelids, Conklin (1910, 1917) in gasteropods. In any instance in which a particular substance may be displaced without modifying normal development, it is evident that we are not dealing with an organ-forming substance. But in every such instance in which typical parts develop from typical regions of the egg it is logically necessary to assume that there is some material substance which remains in its original position and in which the typical pattern persists, unless we are willing at once to go to the realm of immaterial things for an explanation.

In a few instances differentiated substances of the unsegmented egg can be shown to be organ-forming, that is, so fully differentiated that without them particular organs will not form, while if they are displaced from their typical positions by pressure or centrifugal force the organs which would normally form from them are also displaced. It is generally true that fragments of an unsegmented egg, above a certain minimal size, are capable of developing into whole embryos. O. and R. Hertwig (1887) found that pieces of the unsegmented egg of echinoderms could segment. Boveri (1889) found that both nucleated and non-nucleated pieces could be fertilized and would develop into larvae. However, Boveri's later work (1901) showed that immediately after maturation and fertilization of *Strongylocentrotus* there is a progressive localization of egg substances, and this has also been observed in nemerteans (Wilson, 1903; Zeleney, 1904), *Dentalium* (Wilson, 1904), fresh-water pulmonates (Conklin, 1911), *Chaetopterus* (Lillie, 1909), ascidians (Conklin, 1905), and the frog (Roux, 1887; Brachet, 1911). In several of these cases it has been proved by experiment that there is a progressive limitation of the potency of parts of the unsegmented egg as it approaches the first cleavage.

In the ascidian *Styela* the material of the yellow crescent is the lightest substance in the egg, the gray yolk is the heaviest, and the clear plasma is intermediate between these two. Immediately after fertilization these substances stratify, the yellow substance collects at the vegetative pole, next above this is the clear plasma, and the gray-yolk substance occupies the animal hemisphere of the egg. If eggs are centrifuged after fertilization they turn within the egg membranes, if the latter are not compressed, so that the vegetative pole is central and the animal pole distal on the centrifuge, and consequently the normal stratification is merely accentuated. But if the eggs are compressed by mutual pressure or within capillary tubes so that they cannot rotate, these three egg substances may be displaced from their normal positions, and if they are so held until after cleavage begins these substances are abnormally distributed to the cleavage cells. Under these circumstances development is always abnormal. In the larvae of ascidians it is possible to recognize by their colors, shapes, sizes, and histological characters the endoderm, chorda, neural plate, and muscle cells, even when these are far from their normal positions, and in the eggs in which the egg substances have been dislocated by centrifuging the larval parts to which they typically give rise are also dislocated. Thus larvae may be turned inside out, the endoderm, muscles, and chorda being on the outside, ectoderm, neural plate cells, and sense organs on the inside; or any other atypical location of these parts may be caused by dislocation of these substances of the unsegmented egg (Fig. 12).

When eggs of *Styela* are centrifuged during the telophase of the first cleavage all the material of the yellow crescent is sometimes forced into one of the two daughter-cells. The cleavage and gastrulation of such eggs is abnormal and the larval muscle cells, which form from the material of the yellow crescent, lie entirely on one side of the median plane. When eggs are centrifuged during the telophase of the third cleavage the yellow material is sometimes forced from the dorsal-posterior into the ventral-posterior cells of the 8-cell stage, and here the yellow muscle cells develop in the ventral hemisphere, and ultimately form a rounded mass on each side of the imperfect larval tail.

These cases are particularly interesting since they demonstrate that in this case the differentiation of muscle cells depends upon the muscle-forming cytoplasm rather than upon the nuclei. Nuclei which would normally have been located in ectoderm cells come to lie in muscle cells, whereas muscle cells develop wherever the yellow material is located. A similar instance has been described by Spemann (1914) in *Triton* where the part of an embryo that develops from part of an egg is shown to depend upon the egg plasm and not upon the nuclei.

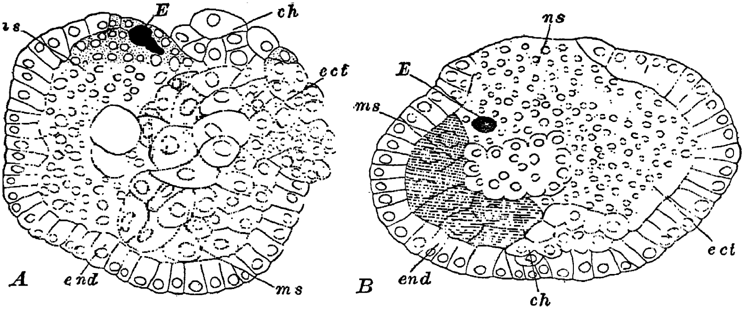

FIG. 12.—Abnormal larvae of *Styela* produced by centrifuging before the first cleavage; endoderm (*end*) on the outside, ectoderm (*ect*) inside, chorda (*ch*), muscles (*ms*), neural plate substance (*ns*), and eye spots (*E*) all displaced from normal positions as a result of the dislocation of corresponding organ-forming substances in the unsegmented egg.

These visibly different substances of the ascidian egg are really "organ-forming," whereas in most other cases the visibly different substances are not organ-forming since they can be dislocated without greatly modifying normal development. However, in all cases in which normal development follows after the dislocation of substances it is logically necessary to assume that there is some substance in which the pattern of the egg persists and which is not moved by centrifuging. There is no possibility of questioning the fact that the areas of cytoplasm in which are located the visibly different oöplasmic substances are in some way different from one another. The orange pigment of the *Strongylocentrotus* egg is not organ-forming, but the mechanism which locates this pigment in a sub-equatorial zone is organ-forming, and this mechanism must reside in some material substance in the egg. The visible localizations of an egg are evidences of the existence of some differential which causes these localizations, and if normal development follows the dislocation of visible substances, it proves not only that these visible substances are not organ-forming, but also that some portion of the cytoplasm in which the substances normally lie is organ-forming.

In short the conclusion expressed with regard to the causes of polarity applies also to egg pattern, namely, it must be found in some substance of the egg which does not change position when the yolk, pigment, and other substances are dislocated, and the only material substances of the egg which fulfil these conditions are the ectoplasmic layer and the spongioplasmic framework.

C. Cleavage and Cell Differentiation

Until the nineties of the last century it was customary to trace the differentiations of development back to the germ layers and there to stop, for at that time few attempts had been made to find still earlier ontogenetic differentiations in the blastomeres or in the unsegmented egg. Cleavage of the egg was regarded as a "mere vegetative duplication of cells" (W. K. Brooks). It is true that Whitman (1878) had shown that specific blastomeres of *Clepsine* developed into specific portions of the embryo, that Roux (1884) had announced that from the 4-cell stage on the frog's egg is a mosaic work of four independently developing pieces, that Chabry (1887) had found isolated blastomeres of *Ascidia* always developing into those parts of the larva which they would produce under normal conditions—but these were considered to be doubtful or exceptional cases, and in general cleavage was regarded as merely a process of cell multiplication and not one of cell differentiation.

And yet the most cursory study of cleavage shows that in the majority of animal classes notable differences are found among blastomeres. These differences concern the size, position, rate of division, and histological character, or quality of contents, of different cells; these differences are usually so constant that it is possible to identify particular cells not only in different individuals and in successive stages of development but also in different species, genera, and even phyla of the animal kingdom.

1. *Cell lineage:*

Beginning with the early nineties, a series of studies was made on what Wilson (1892) aptly termed "cell lineage." The cleavage of the egg in different species of annelids, gasteropods, lamellibranchs, and platodes was studied in great detail, and the striking fact was revealed that in all of these groups the axial relations of egg and embryo, the general form of the cleavage, and especially the origin and destiny of particular blastomeres was constant for each species and closely similar in different classes and phyla. In all of these classes the first two cleavages are meridional; generally the first cleavage is approximately transverse to the median plane of the egg while the second lies in or near the median plane. Four cells are thus formed which are usually known as macromeres. Where there is much yolk the third, fourth, and fifth cleavages are very unequal, the small cells or micromeres lying at the animal pole and the larger cells, the macromeres, at the vegetative pole. Three sets of micromeres are separated from the macromeres, and these three sets are known as the first, second, and third quartets, each consisting of four cells, one derived from each of the macromeres. Where there is little yolk there is little difference in the size of "micromeres" and "macromeres," but the destiny of these cells is the same irrespective of relative size. A fourth quartet formed from the macromeres differs notably in size and contents from the first three quartets, and the left posterior member of the fourth quartet (4*d*) differs greatly from the other members (4*a*, 4*b*, 4*c*).

I remember with what amazement Wilson and I found in comparing in 1891 our results on the development of *Nereis* and *Crepidula* that in both of these animals, representing different phyla, the directions and axial relations of successive cleavages were practically the same; that the ectoderm invariably came from the first three quartets; that the mesoderm came from the left posterior member of the fourth quartet (4*d*), and that all the remaining blastomeres gave rise to endoderm. Later it was found that these conditions occur in all annelids, gasteropods, lamellibranchs, polyclades, and nemerteans, and in addition many other detailed resemblances were discovered. For example, it was found that the apical sense organ, when present, comes from the cells lying nearest the animal pole, that the cerebral ganglia come from the "anterior rosette cells," that the prototroch and velum come from the same region and in part from the same cells in both annelids and mollusks, that the cell which gives rise to the mesoderm of the adult (4*d*) also contains endodermal elements, and hence is a "mesentoblast," that in all lamellibranchs, gasteropods, and polyclades additional mesoderm called by Lillie (1895) "larval mesoderm," because it contributes to the formation of larval organs, is derived from the second or third quartet of ectomeres. The work of Lang (1884) on the polyclades of the Gulf of Naples had indicated that the ectoderm came entirely from the first quartet of micromeres and the mesoderm from the second quartet, but the later work of Wilson (1898) proved that here also the ectoderm is derived from the first three quartets and a portion of the mesoderm ("larval mesoderm" or "ecto-mesoderm") from the second quartet. Finally, Surface (1908) confirmed these findings of Wilson and in addition proved that most of the mesoderm comes from the cell 4*d* which also gives rise to the endoderm and is therefore a "mesentoblast." In all of these respects the cleavage of the eggs of polyclades is brought into line with that of annelids and mollusks (Fig. 13).

Even in species where eggs and blastomeres differ greatly in size and appearance these resemblances in cell lineage are nevertheless present. Thus the egg of *Cummingia tellinoides* is less than one-fiftieth of the volume of the egg of *Crepidula plana*, and there are only insignificant differences in the sizes of "micromeres" and "macromeres," and yet the origin and destiny of the early cleavage cells is identical in these two species. On the other hand, the egg of *Fulgur carica* is about two thousand times the volume of the egg of *Crepidula plana*, and there are corresponding differences in the sizes of certain blastomeres in these two species, and yet the differentiations of cleavage and the destiny of individual blastomeres up to the 50–60 cell stage is cell for cell the same in the two (Conklin, 1907). McMurrich (1886) supposed that the number of the micromeres separated from the macromeres was proportional to the size of the latter, and that in *Fulgur* micromeres continued to form for a much longer time

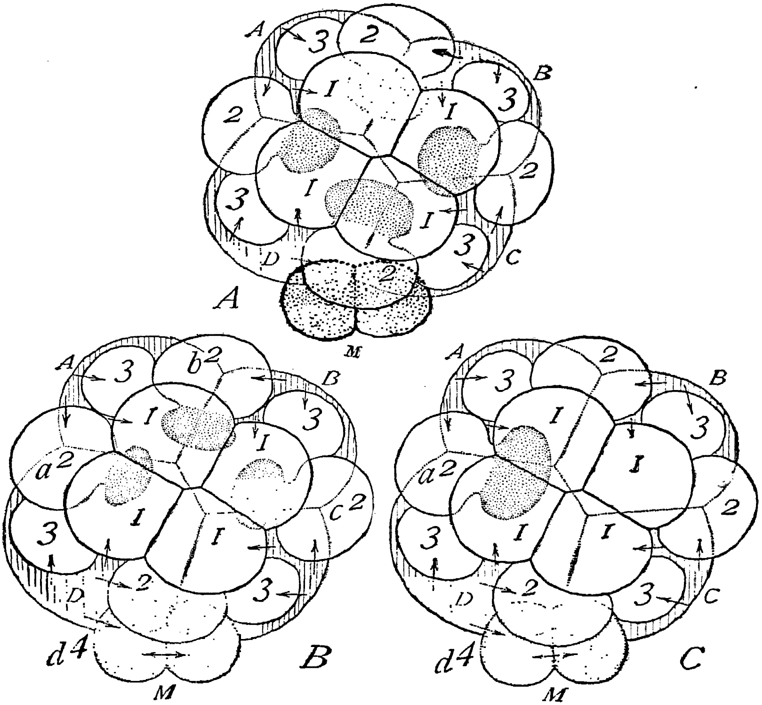

FIG. 13.—Corresponding stages in the cleavage of the egg of (*A*) polyclade (*Leptoplana*); (*B*) gasteropod (*Crepidula*); (*C*) lamellibranch (*Unio*). The first, second, and third quartets are numbered 1, 2, and 3; the macromeres (*A*, *B*, *C*, and *D*); mesentoblast (*M*) or (*d*4); arrows indicate the positions of the spindles in the formation of the micromeres; the stippled inner ends of the second quartet cells represent the "larval" mesoblast (after Wilson).

than in smaller eggs. A similar conclusion was expressed by Fujita (1895) and Viguier (1898). Careful study, however, has shown that this is never true, and that in all annelids, gasteropods, lamellibranchs, and polyclades three and only three quartets of micromeres (ectomeres) are formed, whatever the size of the egg may be (Fig. 13).

The subdivisions of these ectomeres are much more numerous in large eggs than in small ones; at the time of the formation of the shell gland there are about four times as many ectomeres in *Fulgur* as in *Crepidula*, while there are probably thirty times as many at the closure of the blastopore. Nevertheless, all these ectomeres in all cases come from three quartets. The number of organs and differentiated parts is the same in *Fulgur* as in *Crepidula*, and the greater number of ectomeres in the former as compared with the latter is due to a larger number of *non-differential* cell divisions. In all cases that have been thoroughly studied the number and character of *differential* cleavages is the same in all animals of the same class. So far as is known these observations apply to the cells of all the germinal layers and to all the cell differentiations in each of these layers. One of the results of this condition is that differences in body size are generally associated with differences in the number of the constituent cells, though there may also be differences in the size of cells. Whenever there are differences between individuals or species in the number of cells of any particular organ or part it may be taken for granted that this difference is due to a larger number of non-differential divisions in one case than in the other; on the other hand, where the number of cells remains constant, differences in the size of parts must be due to differences in the size of the constituent cells (Conklin, 1912).

In other phyla of the animal kingdom, notably ctenophores, rotifers, nematodes, and ascidians, other types of cleavage and differentiation occur, but the constancy of relation between cleavage and differentiation is just as notable here as in the groups previously named. Indeed, in some nematodes and ascidians the differentiations of cleavage are more easily seen than in any other animals, and development is in a striking degree a visible mosaic work. For example, in the ascidian *Styela partita* (Conklin, 1905) the cleavage bears a very constant relation to the localization of substances in the unsegmented egg. The first cleavage divides the yellow and gray crescents together with the clear and the yolk-laden plasm into precisely equal right and left halves (Fig. 5 *F*). The second cleavage separates the anterior half of the egg with its gray crescent from the posterior half with its yellow crescent, except that the tips of the horns of the yellow crescent are left in the anterior quadrants (Fig. 7 *A*). The third cleavage is equatorial and leaves most of the clear cytoplasm in the four cells at the animal (ventral) pole, while most of the yolk-laden cytoplasm is left in the four cells at the vegetative (dorsal) pole; the whole of the yellow crescent is left in the dorsal cells, while about half of the gray crescent goes into the dorsal-anterior and half into the ventral-anterior cells (Fig. 7 *B*). In succeeding cleavages the isolation of the different oöplasmic substances, in particular blastomeres, becomes more and more complete, but not until the 22-cell stage is all the substance of the yellow crescent segregated into specific cells, while the substance of the gray crescent does not become isolated in particular cells until the 44-cell stage (Fig. 7 *E, F*). The yellow crescent gives rise to both muscles and mesenchyme, and the material which goes into the muscle cells is more deeply pigmented and contains more mitochondria than that which goes into the mesenchyme cells; these two constituents of the yellow crescent are not segregated into separate cells until the 76-cell stage; the gray crescent becomes the chorda and the neural plate, but these two are not completely segregated in separate cells until the 64-cell stage (Fig. 8). It is plain, therefore, that although these crescents are clearly marked at the time of the first cleavage their substances are not completely segregated into specific cells until much later. The cleavage planes of the ascidian egg are very constant in position, but the early cleavage furrows do not follow closely the lines separating oöplasmic substances.

The fact that cleavage planes do not always follow the boundaries between oöplasmic substances or areas is still more notable in the frog's egg. Here also there is a gray crescent around the anterior side of the egg which gives rise to the chorda and the neural plate, and in about 70 per cent of all cases it is divided bilaterally by the first cleavage. But the first cleavage furrow may cut across this plane of bilateral symmetry at any angle up to 90°, and yet the later development may be perfectly normal (Brachet, 1904). Furthermore, Driesch, O. Hertwig, Schultze, Morgan, and others have shown that the positions of cleavage planes of the frog's egg may be changed by pressure or other means without interfering with normal development. From these facts it is evident that the cleavage does not determine the localization of substances nor the pattern of egg or embryo but that these antedate cleavage. In the case of the frog's egg this pattern may be cut up in various directions and individual blastomeres of particular stages may have various developmental values without interfering with normal development.

In gasteropod eggs the relation between cleavage and differentiation is more constant than in the frog's egg; nevertheless, cleavage planes may be turned out of their normal positions and yet development remain normal; the number of macromeres may be increased from four to six or eight or it may be decreased to two or three and yet each macromere will give rise to three micromeres (ectomeres), and in the end a normal larva may be formed (Conklin, 1912). These facts show that normal development does not depend upon a specific number and succession of cleavages in definite positions but rather upon an egg pattern which may be cut up by the cleavage furrows in various ways without destroying the pattern or the normal results of development. However, if the first or second cleavage is forced into an equatorial position, subsequent cleavage and development are quite abnormal (Fig. 4 *F*).

In the ascidian, on the other hand, it has not been possible as yet to alter the position and direction of the cleavage planes without seriously interfering with normal development. If the first cleavage is turned out of the plane of bilateral symmetry, normal development never follows, and the same is true if any of the first three or four cleavages are forced out of their normal positions. There is evidently here a much more constant relation between cleavage and differentiation than in the frog's egg or the gasteropod egg. It is noticeable that the pattern of differentiated areas of the egg is much more sharply marked in the ascidian than in the frog or gasteropod, and this may be one reason why the cleavage is more fixed, but the chief reason must be found in the fact that the mitotic spindles in the ascidian egg cannot be turned out of their normal positions without seriously modifying the pattern of the egg cytoplasm.

In cnidarians, echinoderms, Amphioxus, and vertebrates in general the typical position of cleavage furrows may be readily altered without destroying normal development. The cleavage in Cnidaria is in some cases extremely inconstant and irregular in the size and position of cleavage cells (see especially Hargitt, 1904, 1906), and yet later development may lead to normal larvae. The egg pattern in this phylum is extremely simple, consisting chiefly of a concentric arrangement of egg substances, with polar differentiation only slightly marked (p. 576) and in whatever position the cleavage furrows may lie every one of the early blastomeres contains portions of all the egg substances and there is no noticeable differentiation of cleavage cells.

In echinoderms the cleavage is typically constant in form. The first and second cleavages are meridional, the third approximately equatorial, and the first eight blastomeres are of nearly the same size. At the 16-cell stage four micromeres appear at the vegetative pole which later give rise to the mesenchyme; above these and below the equator are four large cells which give rise to endoderm, while the eight cells of the animal hemisphere become ectoderm. It had been held generally that the first differentiation of blastomeres in the echinoderms occurred with the formation of the micromeres in the 16-cell stage, until Boveri (1901) showed that the substances of the egg of *Strongylocentrotus* become stratified into three zones before cleavage begins, and that the subequatorial pigmented zone goes into the four large endoderm cells, the non-pigmented zone at the vegetative pole goes into the four micromeres which become mesenchyme, while the non-pigmented zone at the animal pole goes into the ectoderm cells. It is plain that there is a differential distribution of these egg substances to the cleavage cells from the animal to the vegetative poles, though no differentiation of cells in any cross-axis can be detected until much later.

The cleavage in Amphioxus is nearly equal and the contents of the early blastomeres are much alike; consequently, it was supposed (Wilson, 1893) that the first visible differentiation took place at the third cleavage. However, Cerfontaine (1906) has shown that the first cleavage normally divides the egg into right and left halves, that the second cleavage separates two smaller anterior blastomeres from the two larger posterior ones, while the third cleavage separates four smaller cells at the animal pole from four larger ones at the vegetative pole. The directions and positions of cleavage planes are not so firmly fixed in Amphioxus as in ascidians, and the cleavage pattern may be altered to a certain extent without preventing normal development.

In all those cases in which the cleavage pattern may be changed without interfering with normal development, it was at first supposed that there was no correlation between cleavage and differentiation, whereas in other cases such correlation is strikingly visible. Consequently, it was suggested (Conklin, 1897) that, with reference to differentiation, two types of cleavage should be recognized, namely (1) the *determinate* type in which cleavage is typically constant with respect to differentiation, and (2) the *indeterminate* type in which such constancy is lacking; these two types have also been called "mosaic eggs" and "regulation eggs." The discovery that there is a specific differentiation pattern in the eggs of practically all animals before cleavage begins has thrown a flood of light upon this whole question. It is now known that there are few if any animals in which the normal cleavage does not bear a constant relation to the differentiation pattern of the egg, and, on the other hand, there are many animal classes in which this typical relation between cleavage and differentiation can be altered without preventing normal development. It is probable therefore that a sharp distinction between determinate and indeterminate types of cleavage is not justified; all types of cleavage are probably determinate under perfectly typical conditions, and in only a few instances (ctenophores, rotifers, nematodes, ascidians) is the constancy of relation between cleavage and differentiation so fixed that it cannot be changed without interfering with normal development.

2. *Prospective value and prospective potency of blastomeres:*

The recognition of the fact that there is a differentiation pattern even in unsegmented eggs has also thrown a flood of light upon another problem which was once much discussed, namely, whether or not blastomeres are really differentiated at all. A great many experiments were made to determine this point, and the conflicting results were very confusing. Most of the earlier experiments were made upon eggs in which the relation between cleavage and differentiation is not fixed (indeterminate type), and in the main they showed that entire embryos or larvae could be obtained from isolated blastomeres; this was held to prove that these blastomeres were not differentiated. The contrary fact that in certain phyla specific blastomeres always gave rise to specific parts of a larva and not to a whole one was explained by O. Hertwig (1892) as follows: "In consequence of the continuity of development every older cell group must arise from a younger cell group and so finally definite parts of the body from definite segment cells." If this means that in these cases of determinate cleavage *any* older cell group may come from *any* younger cell group, it is not true. If, on the other hand, this oracular phrase, "continuity of development," means continuity of differentiation, namely, that every later differentiation must have arisen from an earlier differentiation and so finally specific differentiations of the body from specific differentiations of blastomeres or of unsegmented eggs, it is in full accord with the latest conclusions based upon the study of cell lineage and egg organization. However, there can be no doubt, as is shown by many other statements, that Hertwig meant to maintain the former of these two alternatives, namely, that particular blastomeres are not differentiated for particular ends.

Roux (1884–91) first undertook to determine by experiment whether the first four blastomeres of the frog's egg were differentiated or not. By killing individual blastomeres with a hot needle in the 2-cell or the 4-cell stage he found that the surviving blastomeres gave rise only to partial embryos. Similar results were obtained by Chabry (1887) in experiments on ascidian eggs. On the other hand, Driesch (1891, 1893 ff.) found that isolated blastomeres of the 2-cell or the 4-cell stage of echinoderms gave rise to whole larvae, and he therefore concluded that cleavage of the egg is a "mere sundering of perfectly homogeneous materials capable of any fate" and that blastomeres might be shuffled about "like balls in a pile" without interfering with normal development. Later work showed that after the third cleavage isolated cells at the animal or vegetative poles would develop to gastrulae but not usually into perfect larvae; however, each of the first two or first four blastomeres of the echinoderm egg is "totipotent" or "equipotent," and its future fate in development is a "function of its position." The cells of the 8-cell stage are more limited in potency than those of the 4-cell stage, and in general there is progressive limitation of the potency of cells as cleavage advances. Driesch admits that in typically normal development specific parts of the body arise from specific blastomeres. This typical or normal destiny of blastomeres he calls their prospective value ("prospectiv Bedeutung"). But he says that under experimental conditions all this may be different; each blastomere may, when isolated, produce an entire embryo, that is, it is "totipotent," and this capacity Driesch calls prospective potency ("prospectiv Potenz").

The fact that in normal development specific parts of the body arise from specific blastomeres implies that *in some respect* these blastomeres are differentiated, and it would probably conduce to clear thinking if this were always spoken of as early differentiation rather than as prospective value ("prospectiv Bedeutung"). Furthermore, since a specific prospective value of a blastomere implies early differentiation, the capacity of such a blastomere to produce other than typical parts or even a whole embryo does not prove that it is undifferentiated; prospective potency is not necessarily a measure of differentiation, it may be a measure of the capacity of regulation, and in that case should be understood to refer to the capacity or incapacity of reversing early differentiations.

As the result of much experimental work it is known that isolated blastomeres are more or less totipotent in the following groups: Medusae with alternation of generations up to the 16-cell stage, in those with direct development up to only the 2- or 4-cell stage but not at the 8-cell stage (Zoja, Maas); nemerteans up to the 4-cell stage but not at the 8-cell stage (Wilson, Zeleny, Yatsu); echinoderms up to the 4- or 8-cell stage (Driesch, Herbst, Loeb, Wilson, Morgan, and many others); Amphioxus in the 2-cell and possibly in the 4-cell stages (Wilson); amphibians in the 2-cell stage, when the first cleavage lies in the plane of bilateral symmetry (Brachet, Spemann).

In the frog's egg, Schultze (1900) found that under certain conditions double embryos could be obtained from a single egg; Morgan (1895) was able to get a whole tadpole from one of the first two blastomeres if the surviving blastomere is inverted so that there is a rearrangement of the egg substances under the influence of gravity. Later Brachet (1904) discovered that either of the first two blastomeres may give rise to a whole larva only when the first cleavage lies in or near the plane of bilateral symmetry. Also in the egg of *Triton* it was found that two whole larvae of half size could sometimes be obtained from the first two blastomeres (Endres, Herlitzka), but Spemann (1918) found that this happened only when the first cleavage furrow lay in or near the plane of bilateral symmetry. When the first furrow is at right angles to the median plane, it divides the egg into dorsal-anterior and ventral-posterior halves; the former of these may give rise to a whole larva but the latter never does. In neither the frog nor *Triton* is any one of the first four blastomeres totipotent.

Driesch repeated the experiments of Chabry on ascidian eggs, and concluded that either of the first two or any one or two of the first four blastomeres would develop into a whole larva. But in *Styela* where every cell of the early cleavage can be easily distinguished by its color and structure from every other one, no single blastomere of any stage ever develops into a whole larva; on the other hand, it invariably gives rise only to those parts of a larva which it would produce normally (Conklin, 1905, 1906). One of the first two blastomeres develops into a right or left half-larva; the two anterior blastomeres of the 4-cell stage develop into an anterior half-larva with neural plate, sense organs, and chorda, but without muscles or tail; the two posterior blastomeres develop into a posterior half-larva with muscles and caudal endoderm cells but without neural plate or chorda; while any one blastomere of the 4-cell stage develops into a corresponding quarter-larva (Fig. 14). I repeated Driesch's experiments on the same species that he used, namely, *Phallusia mamillata*, and found that here also the development of single blastomeres is invariably partial (Conklin, 1911). The same results were obtained in experiments on the eggs of *Ciona* and *Molgula*. All these experiments prove that the visible differentiations of blastomeres in ascidian eggs are real morphogenetic differentiations and that under the conditions of the experiments these differentiations are not reversible.

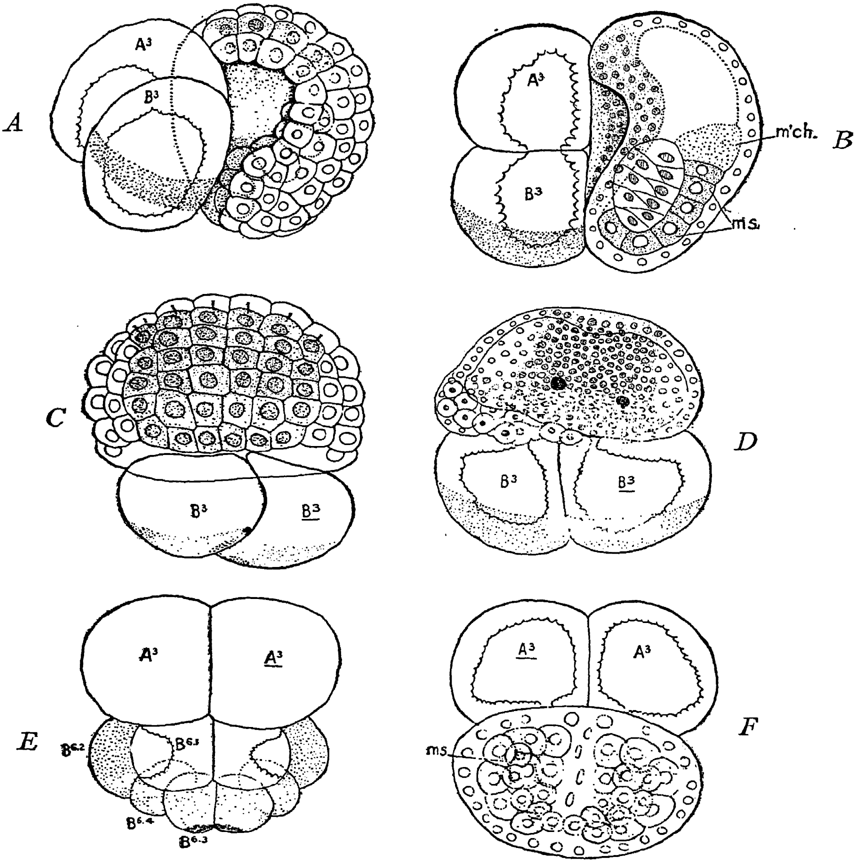

Fig. 14.—Mosaic development of *Styela;* dorsal views of partial embryos. *A*, Right half gastrula with half neural plate, chorda, muscle cells, etc; blastomeres *A*3, *B*3 of the left half having been killed in the 4-cell stage. *B*, Right half larva with neural plate and notochord of half size but with mesenchyme and muscle cells of the right side only. *C*, Anterior half gastrula with all neural plate and chorda cells but with no muscle cells; the posterior blastomeres *B*3, *B*3 having been killed in the 4-cell stage. *D*, Anterior half larva with neural plate, eye spots, and scattered chorda cells, but without any of the parts that develop from the two posterior cells which were killed in the four-cell stage. *E*, Anterior cells killed in the four-cell stage; posterior cells have segmented normally, giving rise to typical yellow crescent cells. *F*, Posterior half of larva from an egg similar to the preceding; muscle cells, mesenchyme, and caudal endoderm present as in normal larva; no trace of neural plate or chorda.

In the case of Amphioxus, Wilson (1893) found that a whole larva would develop from one of the first two blastomeres, and apparently from any two of the first four blastomeres. In one or two instances even a single one of the first four blastomeres was said to develop into a whole larva. I have repeated these experiments, and can confirm Wilson's statement that an isolated blastomere of the 2-cell stage will develop into a whole larva, but I have not been able to get whole development from every individual blastomere of the 4-cell stage. In short, the potencies of the blastomeres of the eggs of Amphioxus, frog, and *Triton* coincide with the segregation of oöplasmic substances. When the first cleavage is in the plane of bilateral symmetry each of the first two blastomeres contains all of the egg substances, and an isolated blastomere of this stage can give rise to a whole larva; but the anterior-dorsal quadrants contain all of the materials which go to form neural plate and chorda, and accordingly a whole larva does not develop from the two ventral-posterior quadrants.

In contrast with the totipotence of any one of the first four blastomeres of medusae, nemerteans, and echinoderms, and in full agreement with the limited potencies of these cells in chordates is the condition found in eggs of ctenophores, nematodes, annelids, and mollusks. In ctenophores, Chun (1880) first observed that isolated blastomeres developed into partial embryos; Driesch and Morgan (1895) confirmed and extended these observations; Ziegler (1898) found that isolated blastomeres gave rise only to those parts which they would have formed if they had remained in their normal relations to other blastomeres; Fischel (1897, 1898) and Yatsu (1911, 1912) have made more accurate and precise studies of the ctenophore egg without modifying in any important respect the general conclusion that the development of isolated blastomeres is strictly partial.

Boveri and Miss Stevens (1909) found that when one of the first two blastomeres of *Ascaris* was killed, the other developed as it would have done under normal conditions. These experiments, added to a great amount of observation on the development of nematodes, demonstrate that in this class of animals cleavage is of the mosaic type.

In the eggs of an annelid, Wilson (1904) found that neither of the first two blastomeres is able, when isolated, to produce a perfect larva, and he concluded that this was due to the presence of "formative stuffs" in the undivided egg which are distributed unsymmetrically to the first two blastomeres.

Crampton (1896) first proved that isolated blastomeres of gasteropod eggs show partial development, and the conclusion was confirmed and extended to other classes of mollusks by Wilson (1904). Detailed studies of the cleavage of isolated blastomeres of *Crepidula* show that the character of development is determined by the "formative stuffs" that they receive (Conklin, 1912).

On reviewing these cases one might conclude that in different classes of animals the early cleavage falls into two more or less distinct types, (1) the indeterminate or regulative type and (2) the determinate or mosaic type. Medusae, nemerteans, echinoderms, Amphioxus, have been assigned to the former of these types; ctenophores, nematodes, annelids, mollusks, ascidians, to the latter; and by different authors at different times amphibians have been assigned to both types. It has generally been assumed that the fundamental difference between these two types lies in the time at which cellular differentiation appears, for in all cases such differentiations appear sooner or later, and in all instances the potencies of individual cells becomes progressively limited as development advances.

But the conflicting results on the potencies of blastomeres in amphibians is due largely to the fact that the cleavage furrows bear no constant relation to the differentiations of the unsegmented egg (Brachet, Spemann); the egg differentiations are approximately constant both in form and time of appearance, but the positions of cleavage planes are not constant. There is no direct evidence that the unsegmented egg of Amphioxus is less highly differentiated than that of an annelid. May not the differences in the potencies of the blastomeres in these animals be due to the positions of the cleavage planes with reference to the differentiations of the egg rather than to variations in the time of appearance of those differentiations?

It is certainly no chance occurrence that in all cases in which blastomeres are totipotent there is equal or symmetrical distribution of formative substances to the cleavage cells, whereas in almost all cases where individual blastomeres lack the capacity of forming a whole organism this distribution is unequal or unsymmetrical. In Medusae, echinoderms, and nemerteans there is no visible indication that any one of the first four blastomeres contains formative materials not found in the other three; in the two last-named groups, however, there is visible differential distribution of substances to the animal and vegetative poles. The developmental potencies of the early cleavage cells correspond with the differential or non-differential distribution of the substances to these cells. In polyclades, rotifers, annelids, and mollusks the first cleavage is nearly transverse to the antero-posterior axis; in nematodes it is nearly midway between the ectodermal and endodermal poles; in all of these cases the formative materials are distributed differentially to the first two blastomeres and consequently development of individual blastomeres is partial. In ctenophores and ascidians these materials are distributed symmetrically to the right and left blastomeres, but in these groups the symmetry is so fixed that individual blastomeres of the 2-cell stage do not establish a new plane of symmetry even though each contains one-half of all the formative stuffs; consequently each develops as a right or left half-embryo. In cases where the anterior and posterior poles of the undivided egg are differentiated, the blastomeres that form at these poles are not totipotent; and probably in all cases where there is a differential localization of substances at the animal and vegetative poles of the egg, the blastomeres at these poles are not capable of developing into an entire organism.

Another factor enters in the question of the potencies of individual blastomeres, namely, the capacity of dedifferentiation and redifferentiation. In the ascidian egg this capacity is very limited, whereas in the adult ascidian it is very considerable. On the other hand, in Amphioxus the right or left blastomere may establish a new plane of symmetry so as to give rise to a whole larva; but in the adult the capacity for regulation is comparatively slight. It seems probable that the greater or smaller capacity for regulation in these cases does not depend so much upon the degree of differentiation as upon the fluidity or lability of the cytoplasm. The ascidian egg is less fluid than that of Amphioxus, whereas the reverse is true of the adult of these two animals. There is no conclusive evidence that the ascidian egg is more highly differentiated than that of Amphioxus, but it is more stable.

D. The Mechanism of Differentiation

In broad outlines embryonic development consists of differentiations built upon preceding differentiations. It begins with the most general features of polarity, symmetry, and pattern of the egg; it then proceeds to the more detailed differentiations of the polarity, symmetry, location, and pattern of constituent cells and parts of the embryo and finally to the differentiations of organs and tissues. The symmetry of limbs (Harrison, 1921), of the auditory labyrinth (Streeter, 1907), and of many other parts is not determined until long after that of the body as a whole; the differentiations of the ectoderm for optic lens or neural plate arise long after the ectoderm is differentiated from the other germ layers, and specific parts of the nerve tube, after the plate as a whole has arisen (Spemann, 1918; Ruud and Spemann, 1922). In short, development is progressive, increasing differentiation in which many of the essential conditions are found in other earlier differentiation. Every differentiation is in this sense correlated with others; and yet there is in many cases a considerable degree of independent or self-differentiation, as is seen for example in the "mosaic" type of eggs.

1. *Cell division and differentiation:*

The most evident differentiations of cells, such as are found in muscle, nerve, and various kinds of connective tissue, as well as in ova and spermatozoa, occur during intervals between cell division or after such divisions have altogether ceased. The differentiations that occur in many Protozoa, as well as in individual tissue cells of Metazoa, demonstrate that such differentiations are not immediately caused by cell division.

But in embryonic development, cell division does play an important part and the ultimate fate of a cell is determined long before its final differentiations become visible; that is, the foundations of future differentiations are laid in very early stages. A certain amount of embryonic differentiation is possible without cleavage, as Lillie (1902) has shown, but there can be no question that a higher degree of specialization is possible in a multicellular than in a unicellular body. However, the number of cells in a body or organ is no measure of the degree of its differentiation, for many cells may be alike; rather it is the number of unlike cells and the extent of their unlikeness that is the measure of differentiation.

Some cleavages of an egg are differential and others non-differential depending upon the unlikeness or likeness of the daughter-cells. Non-differential divisions serve to reduce the size and to increase the number of cells; differential divisions also segregate and isolate different cytoplasmic substances in different cells. The fission of a protozoan, such as *Paramoecium*, is, in its early stages, differential; but as division progresses it is accompanied by a process of regulation which tends to make the daughter-cells alike. But in the cleavage of an egg, such regulation is lacking, in typical conditions, and initial differences between daughter-cells persist and tend to increase.

At the moment of division daughter-cells may differ in size, quality, and relative position; they may differ later in direction and rate of subsequent divisions and increasingly in quality of cell contents. Probably the intrinsic causes of all these cell differentiations are fundamentally the same, namely, the localization of different substances in particular parts of cells, and the orientations of division planes with reference to these localizations. Given a cell which is not homogeneous, or in which every radius is not like every other one, it follows that cell division will be differential or non-differential depending upon the position of the cleavage planes. Under normal conditions these planes are constant in position and they follow one another in a definite sequence. Consequently, the character and pattern of the cleavage and its relation to differentiation is nearly constant for each species. Any explanation of the causes of differential cell division must account for the localizations of different materials in cells and for the orientations of the division planes.

a) CYTOPLASMIC LOCALIZATION

We have seen that in the maturation and fertilization of the egg there are active movements of the ectoplasm and spongioplasm by which different substances become stratified in the chief axis and localized in different cross-axes. Similar movements continue throughout the cleavage, becoming less striking as the cells decrease in size. These movements are always most active during the period of mitosis; they begin with the early prophase and especially with the dissolution of the nuclear membrane, and continue until the following telophase. The principal facts as to these movements during cleavage may be summarized as follows: (1) With the escape of nuclear sap into the cell body at the beginning of mitosis, rotary or vortical movements are set up around the two poles of the spindle as centers. (2) The vortices at the two poles of the spindle are symmetrical, and the rotations in the daughter-cells are in opposite directions. (3) The tension of the ectoplasmic layer opposite the poles of the spindle decreases, and lobes may form at these places either normally or when the eggs are subjected to pressure. (4) Division walls form where vortices meet (Fig. 15). (5) Other movements, presumably due to the ectoplasm and the spongioplasmic framework, lead to the stratification or localization of different cell contents in particular parts of cells, and to the orientation of mitotic spindles. These movements of orientation differ in successive cleavages but are typically constant for each cleavage; they are the causes of all differential cleavages.

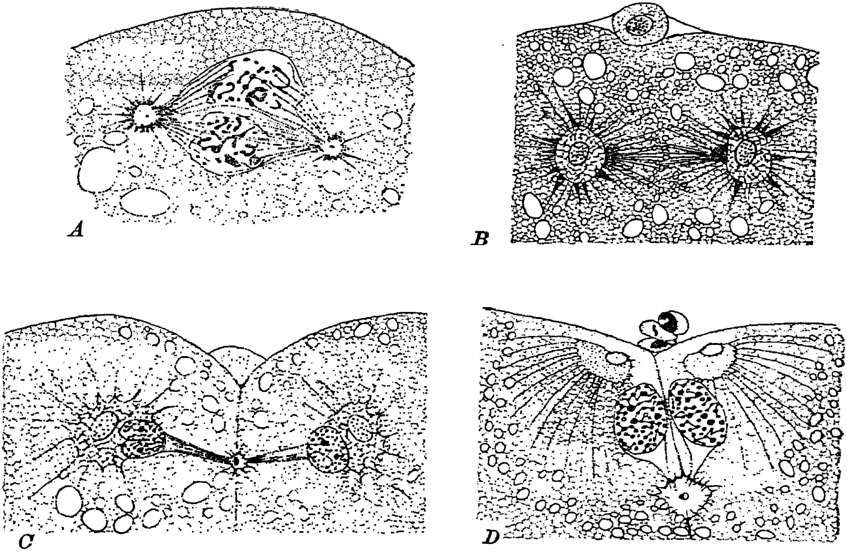

FIG. 15.—Sections of successive stages in the first cleavage of *Crepidula*. *A*, Prophase of first cleavage; egg nucleus above, sperm nucleus below; granules of oxychromatin on spindle fibers. *B*, Anaphase; ring-shaped centrosomes within large centrospheres at the poles of the spindle; spindle axis a straight line. *C*, Telophase; centrospheres and centrosomes turning toward the surface; mid-body at middle of spindle, away from the surface; spindle axis beginning to bend on itself. *D*, Two cells; resting stage; centrosomes and spheres near polar bodies; mid-body near middle of egg; spindle axis doubled on itself; clear ectoplasm carried down from surface along partition wall.

b) THE ORIENTATIONS OF CLEAVAGE

One of the simplest forms of differential cleavage is that in which daughter-cells are unequal in size. Mere difference in size may not indicate difference in quality, and daughter-cells which are quantitatively unequal may have equal developmental potencies. But in many, perhaps most, instances difference in size of daughter-cells is associated with differences in quality of contents.

What is the mechanism of unequal as contrasted with equal cell division? Evidently it is associated with the position of the mitotic spindle, for the partition wall between daughter-cells invariably passes through the middle of the spindle, and if the spindle stands in the middle of a cell, division will be equal; if it is eccentric, division will be unequal. The most unequal of all cell divisions are those by which the polar bodies are separated from the egg. In *Crepidula plana* the relative volumes of first polar body and egg are 1:1,442, in *Fulgur carica* 1:1,211,355; in some species the differences are not quite so great, in others much greater; but in all cases, typical polar bodies are many times smaller than the egg. Careful study of the first maturation division of *Crepidula* shows that when the spindle first appears it may form at any angle with the egg axis, and it lies near the middle of the egg; if the division wall were to form while the spindle is in this position, a polar body would be given off whose diameter would be to that of the egg as 1:2 or 1:3. Later the spindle turns into the egg axis and moves toward the periphery until one pole comes into contact with the cell membrane. The membrane then protrudes over this pole and the end of the spindle projects into this protrusion; at the same time the spindle itself constantly grows shorter until finally it is not more than one-half its original length, and when the division wall finally passes through the equator of the spindle, it separates a polar body from an egg which is more than 1,400 times its size.

The principal factors in this unequal division are the orientation of the spindle in the egg axis, its movement to the periphery, its shortening and protrusion. Both observation and experiment demonstrate that in all these respects the spindle is passive; it is oriented and moved and shortened by activities outside itself. Following the dissolution of the membrane of the germinal vesicle and the escape of nuclear material into the cell body, active movements are set up in the latter which turn the spindle into the egg axis, thrust one end of it against the cell wall at the animal pole, and cause it to protrude into a lobe which is formed at this point.

Similar conditions are found in all unequal cleavages of the egg. The first cleavage is sometimes unequal, as, for example, in *Nereis* (Wilson) and *Unio* (Lillie); in the latter case Lillie (1901) found that the mitotic figure when first formed stood near the middle of the egg; it then moved into an eccentric position, and after oscillating back and forth came to rest with one pole in contact with the periphery of the egg.

In nearly all polyclades, annelids, and mollusks, the third, fourth, and fifth cleavages, by which the three quartets of micromeres are formed, are unequal. The causes of these unequal divisions are like those already described, namely, the orientation of the spindle and its movement into an eccentric position. In *Crepidula* a lobe of cytoplasm containing a clear fluid forms in the position of the future micromere (Fig. 16).

It is also plain that the cause of differential cleavage cannot be found in differences in the poles of the spindle or in the two daughter-nuclei. It is true that the peripheral aster becomes smaller than the central one, but this is only

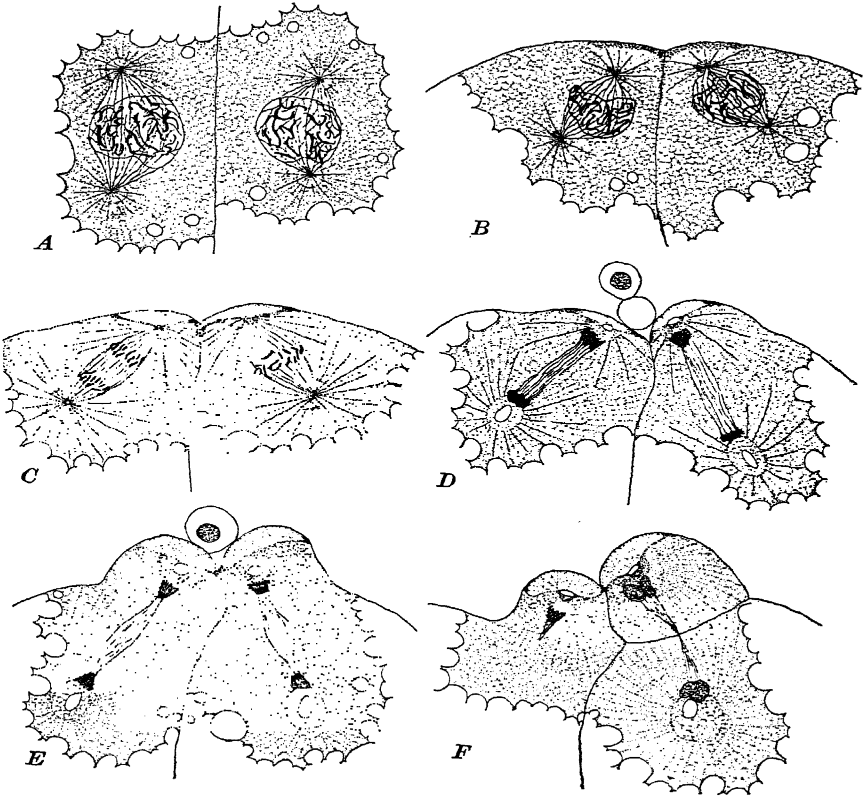

FIG. 16.—Sections of mitotic figures of the second and third cleavages of *Crepidula*. *A*, Prophase of second cleavage; mitotic figures showing oxychromatin granules on spindle fibers and with both poles of each spindle equidistant from the cell surface. *B–F*, Successive stages in the third cleavage; sections cut in meridional plane; spindles oriented with one pole toward the animal pole of the egg, here marked by granular sphere substance derived from previous mitosis. *B*, Prophase; upper pole of spindle covered by granular sphere substance. *C*, Metaphase and anaphase; sphere substance at upper pole spread into a ring within which is clear ectoplasm which is bulging to form a lobe over end of spindle; centrosomes at two poles of spindle approximately equal in size. *D*, Anaphase; further lobing of the cytoplasm over upper end of the spindle; centrosomes at upper pole smaller than at lower. *E*, Late anaphase; lobes over the upper end of the spindle still more prominent with clear ectoplasm forming a disk within the granular ring of sphere substance. *F*, Telophase showing separation of micromere from macromere, partition wall having passed through middle of spindle; disk of ectoplasm very prominent over upper end of spindle; double nuclei in each cell represent derivatives of egg and sperm nucleus; size of centrosomes and spheres at the two poles proportional to volume of cytoplasm in which they lie.

after it has taken a peripheral position; when the spindle is first formed near the middle of the cell both asters are equal in size. Furthermore, it is possible by centrifugal force to drive a maturation spindle through the egg and cause a polar body to be formed at some other place than the animal pole; in many such cases the aster and daughter-nucleus that go into the polar body are the ones that would have remained in the egg under normal conditions; while the ones that remain in the egg are those that would normally have gone into the polar body—and yet perfectly normal development may follow. This proves that the cause of unequal cell division is not to be found in spindle, aster, or nucleus. Differences in the sizes of these structures are the results rather than the causes of unequal cell division.

It has generally been assumed that equal cleavages, alternately at right angles, are due to simple mechanical conditions, such as the greatest diameter of the cytoplasm, or the direction of least resistance, and that they require no further explanation (O. Hertwig, 1892). As a matter of fact, equal cleavages and successively alternating ones cannot be explained in so simple a manner. The fact that the first cleavage spindle in *Crepidula*, for example, stands at right angles to the chief axis of the egg and with its equator in that axis shows that there is here some orienting power of the highest significance. There may be considerable variation in the positions of the cleavage centrosome and in the initial position of the first cleavage spindle without any corresponding variation in the final position of the spindle or of the cleavage plane. It seems necessary to conclude that in every cleavage, whether equal or unequal, polar, meridional, equatorial, or whatever its direction may be, there is some orienting mechanism which differs in different cleavages, and yet in all cases is located in the activities of the cytoplasm.

In the structure of the egg of *Crepidula* no other organization has been discovered which would explain the fact that the first two cleavages are equal, the three following ones very unequal, and subsequent cleavages more nearly equal again, except the aggregations and movements of the cytoplasm, and particularly of that more viscid portion of the cytoplasm, the ectoplasm and spongioplasm.

It has usually been supposed that the inequality of macromeres and micromeres is due to the quantity of yolk contained in the former, and where the quantity of yolk is great this is undoubtedly one of the factors involved; but that it is not the only or even the principal factor is indicated by experiments in which by centrifuging eggs of *Crepidula* at the first or second cleavage one or two of the macromeres come to contain no yolk; in those macromeres which contain no yolk subsequent divisions are still unequal, protoplasmic micromeres of the usual size being separated from the protoplasmic macromeres (Fig. 4*E, F*). Among the protoplasmic micromeres, certain cell divisions are quite unequal; for example, one daughter-cell may be eight times the volume of the other at the time of division; yet here there can be no possibility that this inequality is due to the presence of yolk, since no yolk is present in either daughter-cell. It might be considered that this inequality was due to mutual pressure among the cells were it not for the fact that in isolated blastomeres the same inequalities of division occur as in the normal cell complex.

In all cases of determinate cleavage the position of the spindle and consequently the direction of division, the relative sizes of daughter-cells, and the quality of those cells are determined by some structural pecularity of the protoplasm, and not by the presence of inclusions within the cell or by pressure from without. The study of normal as well as of artificially altered cleavage of *Crepidula* points unmistakably to the conclusion that the definite position and axis of each spindle is fixed by the structure of the cell protoplasm, and since the position and axis of the spindle change regularly in successive divisions this protoplasmic structure must change regularly in successive cell generations.

All that has been said regarding the causes of unequal cleavage applies with equal force to other types of cell division. Evidently the positions of cells in the cell complex are determined to a great extent by the position and direction of cleavage planes. Likewise, the quality or material contents of daughter-cells depends upon the localization of different substances in cells and the orientation of division planes with respect to such localizations. Obviously varying rates of growth and division of daughter-cells depend to a great extent upon the quality of those cells.

c) FUNCTION OF PARTITION WALLS IN CELL DIFFERENTIATION

Although a certain amount of differentiation of an egg is possible without cleavage (Lillie, 1902) such differentiation is limited largely to the formation of cilia in certain areas. Progressive differentiation of development is dependent upon the progressive segregation and *isolation* of different substances. This is clearly shown by the development of eggs in which the formation of certain cleavage furrows has been suppressed without stopping subsequent nuclear and cell divisions. Such suppression of partition walls between cells without suppression of nuclear division is easily brought about by shaking, pressure, increased temperature, hypertonic or hypotonic solutions, chemicals, etc. After such eggs have been returned to normal conditions they may go on in their development without the missing partition wall ever being formed. If this partition wall in normal eggs separated differentiated cells, its absence leads to a commingling of the cytoplasm of the two halves and to the absence of all differentiation between the cells derived later from these two halves. Within the same cell body, two mitoses are simultaneous, and the resulting daughter-cells are similar. The importance of division walls for the isolation of morphogenetically and chemically different substances in differentiation is thus clearly indicated (Conklin, 1912).

2. *Chromosomal heredity and cytoplasmic differentiation:*

The evidence is conclusive (1) that genes, or Mendelian factors, are located in the chromosomes; (2) that in mitosis each chromosome, and presumably each gene, divides equally and the halves are distributed equally to the daughter-cells; (3) that consequently all cells of an organism have typically the same chromosomal constitution, unless the chromosomes become secondarily modified by different kinds of cytoplasm or different environmental influences. How then is it possible to correlate chromosomal inheritance and cytoplasmic differentiation? How can identical genes in every cell lead to the multitudes of inherited differences in differentiated cells?

It is very significant that although chromosomes always divide with exact equality, the cell body does not. Owing to the fact that the egg cytoplasm is not isotropic but contains different substances in different axes and areas it follows that the cytoplasm of the daughter-cells frequently differs, though the chromosomes are the same in all cells, and the same chromosomes and genes interacting with different kinds of cytoplasm produce different results.

Differential cell division is the result of definite movements of the cytoplasm, of definite orientations of spindles and cleavage planes, and ultimately of a definite polarity, symmetry, and pattern of the cytoplasm. There is good evidence that these movements, orientations, and localizations in the egg are the immediate results of cytoplasmic activity; these activities may themselves be the results of the interaction of nucleus and cytoplasm at an earlier stage, and possibly the inherited differential for all these orientations of development may be found in chromosomes or genes. Toyama (1913), Tanaka (1916), and Uda (1923) have shown that certain characters of the unfertilized eggs of silkworms are inherited in Mendelian fashion, and this indicates that they are inherited through chromosomes. It seems probable that some of the inherited factors that determine egg pattern, and perhaps even the polarity and symmetry of the egg, may be located in the chromosomes of the oögonia and oöcytes. The fully formed germ cells have specific, inherited differentiations, and it seems most probable that these differentiations have arisen just as the differentiations of tissue cells have, namely, under the influence of particular genes carried in the chromosomes acting upon specific kinds of cytoplasm. But it is impossible to prove that the cytoplasm of any cell at any stage of its differentiation functions merely as food or as a homogeneous medium for the nucleus. Undoubtedly, chromosomes are factors in determining the type of organization of an egg or spermatozoön, just as they are in determining the organization of any tissue cell, but they are not the only factors.

Given a definite polarity, symmetry, and pattern of the egg, all other differentiations of ontogeny could be explained as due to the interaction of non-differentiating genes on different parts of this cytoplasm; but there is no mechanism by which embryonic differentiations could come from the action of non-differentiating genes on a homogeneous cytoplasm. The genes or Mendelian factors are undoubtedly located in the chromosomes, and they are sometimes regarded as the only differential factors of development, but if this were true these genes would of necessity have to undergo differential division and distribution to the cleavage cells, as Weismann maintained. Since this is not true it must be that some of the differential factors of development lie outside of the nucleus, and if they are inherited, as most of these early differentiations are, they must lie in the cytoplasm.

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SECTION X

THE CHROMOSOME THEORY OF HEREDITY

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THE CHROMOSOME THEORY OF HEREDITY

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The chromosome theory of heredity is a specific development of the more general one which postulates the existence within the organism of a special substance, the function of which is to transmit and control the development of characters peculiar to the group. At first this "idioplasm" was a purely theoretical one, but, as a result of observations upon the intimate details of cell division by mitosis, Wilhelm Roux (1883) was led to the conception that the nuclear chromatin possesses the attributes which theoretically should belong to an idioplasm. Taking this suggestion as a basis, Weismann (1886, 1894) erected a highly theoretical superstructure which went far beyond the observed conditions of the chromatin. Because, in the definiteness and precision of their structure and behavior, the chromosomes most strongly suggest a hereditary mechanism, the theory relating to the function of the chromatin has come to be known as the "chromosome theory of heredity."

Before attempting an account of this theory it will be well to come to a definite understanding of the meaning of the terms employed. Concerning the substance "chromatin," we have little definite knowledge, so far as its chemical character and transformations are involved. The staining reactions by which it is demonstrated in our preparations are not chemical tests of precision, but *they do indicate for the chromatin an extensive and exact series of changes* intimately and specifically related to the activities of the cell.

It is of first importance to note that the chromatin has a definite architecture and disposition within the nucleus, and that it obviously dominates here. While there are no specific chemical tests for chromatin, and despite the appearance in the cytosome of substances that stain similarly, it seems assured that, so long as a karyotheca exists, the chromatin as such is confined to the nucleus. Obviously there are material interchanges between the two regions of the cell, but that the chromatin in its peculiar morphological character fragments and passes out of the nucleus seems very doubtful.

That there are two varieties of chromatin, oxychromatin and basichromatin, is uncertain. This distinction has been established entirely upon the differences in staining reaction at various periods of nuclear activity. Strictly correlated with these variations in color reactions are definite physical conditions of the chromatin and, within a limited region, it is possible to see the acid and basic dyes individually retained, thus marking the degree of concentration —the condensed, homogeneous chromatin showing as basichromatin, while the diffuse portion stains as oxychromatin.

The distinction also between a linin reticulum and a supported chromatin rests upon a very insecure observational basis. It is clear enough that in different regions of the nuclear thread there is a variable reaction to stains. Commonly, the thinner, more extended portions stain lightly or not at all, while grosser masses react strongly. That these differences mark the presence of two distinct substances is, however, very doubtful. It is much more reasonable to conclude that observable differences of appearance in the chromatin are due to its varying states rather than to admixtures of substances.

But in gaining an understanding of the nature of the chromatin it is necessary, above all, to keep clearly in mind the physical characteristics of the material. In the living condition it is not a solid substance moved about by extraneous forces, but a semi-fluid colloid, capable of a wide range of intrinsically controlled movements. These changes of form and position are obviously attended by liquefactions and gelations of a definite and limited order. Upon these the microtechnical processes used have a variety of effects and require for their interpretation the greatest care. The proportion of the elements in the fixing fluid, their concentration, temperature, time of action, and many other circumstances have pronounced and variable effects. Even the method by which the animal is killed strongly modifies the resultant picture in the mounted preparation.

While much remains to be done before we can hope to understand the delicate and involved nature of the nuclear structures, our present knowledge, empirical as it is, is sufficient to permit us to say that these constitute a continuous system. In preparations of a high order of technical excellence it is possible to see that the chromatin is directly continuous with the nuclear matrix. In turn it may be noted that there exists no break between nucleus and cytosome, but that there is direct continuity between the substances of these two cellular regions marked only by line of greater concentration at the delicate karyotheca.

Such a picture is in great contrast to that presented in many cytological articles where it would appear that the chromatin is a sharply delimited substance, often standing practically alone in the nucleus. It is apparent enough to one experienced in these matters that the technical processes employed in such cases were unsuited in their nature and application to the material, and that all other nuclear substances have been dissolved, or precipitated upon the denser chromatin. Above all, it must be noted that this organic continuity is most complete and exact in the growth period of the germ cells where so often it is reported lacking. This period is of the greatest significance in heredity, being the time when the germ cells complete the reaction of the parental substances and prepare them for their segregation in anticipation of a recombination in the next generation. Here it is clear that the continuity of the chromatin with other nuclear materials is most intimate. It has already been noted that this condition is maintained in the relation between nucleus and cytosome, but it goes even farther, for at one period in the prophase of the first spermatocyte of the Orthoptera all the cells of a cyst are bound together by interdigitating processes which penetrate even to the nuclei of adjoining cells. By this means the chromatin of all these cells, which, indeed, are the descendants of a single primary spermatogonium, is in definite organic connection, almost as though the cyst were a syncytium.

Upon the dissolution of the karyotheca in mitosis the chromatin becomes condensed and homogeneous, and in this condition displays most clearly the nature of its organization into unit structures—the chromosomes, which, in every species, appear in a definite series marked by constancy of number, size, form, and behavior. At this time all these features are most strongly apparent; hence the theory associating the chromatin with the idioplasm is known as the chromosome theory of heredity, but there is no doubt that in every stage the same constancy of organization prevails. Indeed, the finest details of chromatin structure are most apparent when the chromosomes are greatly extended, and are less conspicuous when they are fully condensed.

It might appear at first thought that in this state of condensation the chromatin has lost its intimate relation to other portions of the cell, but this is not so. By means of certain fibers, so called, located at a definite point on each chromosome, organic contact is maintained with the non-chromatic part of the cell. This is one of the strongest evidences of precision in cellular organization that we have.

From what has been stated it is certain that the chromatin is an intimate part of the cell, and that whatever functions it performs must be as one member of the complex of parts. It is equally apparent, on the other hand, that in regard to these features in the germ cells, where the activities of each element are of the utmost significance, the behavior of the chromatin becomes compelling in its suggestiveness, especially when considered in relation to genetic phenomena. It cannot be made too clear, however, that no one familiar with the circumstances has a thought of imputing to the chromatin any transcendental function at variance with its position in the cell. It is distinctive in its structure and observable behavior—it must perform a distinctive function. Such a statement gives no warrant for the imputation that the chromosome theory of heredity, which defines this function in part, assumes for the chromatin any independent or uncorrelated influence.

To define our conception of the chromosomes, dealing as it does largely with objective conditions, is much easier than to express an interpretation of the abstract term heredity. This is very evident when one seeks to discover what is the common conception held regarding it. It appears from definitions variously given that heredity is at once a "law," "rule," "force," "material contribution," "act," "relation," "process," "fact," "principle," "link," and "organization." Little wonder that discussions of the subject are so lacking in clearness and precision when the central conception is so poorly defined. An outstanding instance of this confusion of ideas is the almost universal practice of contrasting heredity and variation as factors in evolution. Since variation is only a measure of the precision of hereditary processes, the subject is merely obscured by conceiving them to be opposing forces. Difficulties are introduced also by inaccurate statements which seem to place the operation of hereditary forces in a position, if not opposed to, at least independent of environmental conditions. The organism cannot be conceived apart from its environment. What it is depends upon the form of its adjustment to the physical conditions under which it must exist. Let either the inner constitution of the organism or its environment differ, in one case as compared with another, and the result will be diversity. Since uniformity is obviously impossible, variability is inevitable. For survival, however, the range of such variation is strictly limited. By variation there is meant departure from a type, and the properties of the type which vary are those of a group and not of an individual. The group is continuous and so is the environment, but individuals are only temporary expressions of the interacting forces. Heredity then is the condition or state which is maintained by a certain balance between the operation of forces or conditions intrinsic in a given protoplasmic organization and the external conditions within which it is placed. It also is continuous and a function primarily of the group and not of the individual. The great diversity in conceptions of heredity, as given in definitions, results from the effort to express this property of the group in terms of the individual. In turn, this confusion of thought probably has come from the misconception that the parent produces the offspring from its own body.

The value of the chromosome theory of heredity is due to the fact that it furnishes clear evidence of this group continuity—a continuity which manifests itself, not in a series of fixed forms, but through the repetition of a determinate series of cyclical changes. Heredity governs the manifestations of this cyclical series.

To present the conception of inheritance which is commonly known as the chromosome theory of heredity, there follows a series of numbered statements in which it is sought to present the facts and interpretations upon which the theory is founded.

For the sake of completeness some general biological principles of great importance are included, but receive slight consideration because they are not of immediate relation to the discussion. Again, other phases of the subject, of primary significance, are briefly alluded to because they are discussed at length in other sections. These considerations leave for extended treatment here those parts of the subject which are largely cytological and most intimately related to the chromosomes themselves. Because of limitations of space the account is based primarily upon the phenomena apparent in orthopteran germ cells. This method is justified by the greater familiarity of the author with this field, and because many of the principles involved have been established through observations on this material. In brief, these are: (1) the constancy of chromosome organization and behavior in a large taxonomic group (McClung, Robertson, Carothers, Nowlin, and others); (2) definite association between a particular chromosome and certain characters (McClung on the accessory chromosome and sex); (3) the parallelism between the behavior of the chromosomes in maturation and fertilization and the behavior of characters in Mendelian inheritance (Sutton); (4) the demonstration of chance segregation of paternal and maternal elements in maturation and chance recombination of homologues in fertilization (Carothers); (5) the persistence of chromosome organization as an evidence of specific taxonomic value (McClung); (6) the definite and constant linear arrangement of the chromomeres in the same chromosome in different individuals of the species (Wenrich).

I. ORGANISMS ARE DERIVED DIRECTLY FROM SIMILAR PRE-EXISTING ORGANISMS

That there is a material continuity between generations is now a well-established fact—no spontaneous generation of life or special creation of a new form has ever been observed. The problems of heredity are therefore definite and material, and hence capable of attack by observation and experiment. The outstanding fact thus revealed is embodied in the common expression "Like produces like." No force with which we are familiar will cause any plants or animals to produce offspring that do not have the main characteristics of the parents. Individuals, in other words, exhibit or embody the characteristics of the group, and continue these into a new generation by the process of reproduction. At the same time it is equally manifest that no two members of a group are ever exactly alike. Any theory of heredity must, therefore, explain this apparent paradox of continuity and diversity.

II. THE MODE OF DERIVATION OF ORGANISMS IS USUALLY BIPARENTAL INHERITANCE, IN WHICH CERTAIN CELLS, THE OVUM OF THE FEMALE AND THE SPERM OF THE MALE, FORM THE MATERIAL LINK. INHERITANCE BECOMES THEN A QUESTION OF THE NATURE AND BEHAVIOR OF THESE CELLS

Reproduction, through which process inheritance occurs, may take place in many ways, but in all the higher forms it is carried out by the combined activities of two sexually differentiated parents. To the production of a new individual each of these contributes a single cell, which, in its essential substance, is microscopic in size. These minute masses of living protoplasm constitute the sole material bridge between successive generations. The fertilized ovum is a new individual, specific to the highest degree and embodying all the possibilities of individual development and racial perpetuation. This single unit is all in all. From one pair of organisms there may come hundreds and even millions of such units, each as definite and specific in its character as the other. Incredibly alike, and yet each individually different!

It was for a long time thought, and some still thus incline, that these cellular links between generations are produced by the parents—that out of their bodies they construct these germ cells in their own likeness. In this sense each organism is a special creation, made anew by some intricate process of reincarnation, and embodying in some marked degree the peculiar individual characters of the parents. Such a conception is not in accord with the facts in many well-established cases where the germ cells are traced back in a continuous series to the fertilized ovum from which all the cells within the organism came. Indeed it may be shown that not only is there such a cellular continuity but the nuclear chromosomes are directly derived in a similar manner, and, where our knowledge is greater, the most minute subdivision of the chromosomes may be traced through the series.

III. OF THE MANY ELEMENTS OF THE CELL IT APPEARS THAT THE NUCLEAR CHROMATIN IS THE "IDIOPLASM"—THE SUBSTANCE PRIMARILY CONCERNED WITH THE HEREDITY PROCESSES

In the study of cellular activities it early became apparent that, while the cell is a functional unit, its various parts have taken on, in a differential manner, characteristic phases of protoplasmic functions. The so-called vegetative functions of perception and movement are clearly cytoplasmic, and certain phases of metabolism also, but in reproduction all observations point to the nucleus as of primary concern. It had long been assumed by Nägeli and others, on purely theoretical grounds, that there must be within the organism a substance, the idioplasm, whose function it is to control the processes of reproduction and, thereby, of inheritance. To Wilhelm Roux the apparent concentration of all movements of the cell parts in mitotic cell division upon the accurate and definite separation of the chromatin into exactly equivalent halves, part by part, suggested convincingly that this element of the cell is of primary importance in inheritance. Studies of certain Protozoa showed that the chromatin is differentiated into two kinds of nuclei, one controlling metabolism primarily, and the other reproduction. From this it was assumed that there are generally present in nuclei a trophochromatin and an idiochromatin, although such a differentiation has not been demonstrated. Numerous experiments piled up increasing proof that the chromatin is essential to the life of the cell.

While all studies pointed to the particular function of the chromatin in cellular reproduction, investigations upon the germ cells disclosed a truly marvelous relation between the structure and behavior of the chromatin and the processes of reproduction that have to do with the perpetuation of the species. A study of the phenomena of fertilization demonstrated that, whereas there is a tremendous discrepancy between the material contribution of the sperm and ovum, the chromosomes contributed by the two parents are identical in number and parallel in observable physical attributes. Correspondingly, it appeared that these cells in preparation for this coming together undergo a like series of changes, so far as their chromosomes are concerned. Later studies continue to add consistently to the evidence that of all the substances in the germ cells only the chromatin possesses the attributes of constancy, proportion, and behavior that are necessary to satisfy the theoretical requirements of an idioplasm. The detailed proof for these statements will appear under the various topics of this discussion.

It should be pointed out in this connection that modern theories of heredity do not require the existence of a separate substance, or idioplasm, in the cell, solely devoted to hereditary processes. Reproduction is a function of protoplasm, dependent like all its other properties upon the presence of nuclear chromatin, and there seems no good reason for assuming the presence of a distinct substance to control its operation. Rather we would think of the parts of the cell operating in definite co-ordination, so that cell reproduction would follow inevitably upon the completion of a certain series of metabolic changes. Since heredity manifests itself as the repetition of a definite order of cellular reproductions, it must therefore be dependent upon the operation of the cellular mechanism in each one of these acts of cell division.

IV. CHROMATIN IS NOT A HOMOGENEOUS SUBSTANCE, BUT IS MADE UP OF DISCRETE, DIFFERENTIATED PARTS OF SPECIFIC FUNCTION

A common feature of many theories of inheritance is the postulated existence of a great number of discrete particles of substance, each of which has control in some way over the development of some particular cells or characters in the developing organism. Of such nature were the gemmules in Darwin's provisional theory of pangenesis, the biophors of Weismann, the physiological units of Spencer, the pangens of De Vries, etc.

All the recent, more exact genetic work upon Mendelian inheritance calls for the presence in the "idioplasm" of these separate and distinct genes or factors. To satisfy these theoretical requirements, therefore, the chromatin must be made up of numerous, and necessarily minute, particles of substance. It is, of course, possible that even were these present they might be so small as to be optically invisible. Any evidence indicative of subdivisions of the chromatin would, however, be presumptive of the correctness of this theory.

It is, therefore, of much interest to find that the chromatin is regularly made up of small aggregates, or granules, called chromomeres. These are most visible at periods of greatest diffusion of the chromatin, and may then be seen as more or less regular spherules definitely arranged within the nucleus. At the time of the metaphase, on the contrary, they become much concentrated and the resulting chromosomes appear entirely homogeneous. Similarly in the head of the sperm, which contains the paternal chromosomes, they are lost to view as, indeed, the chromosomes themselves are. Nevertheless, the chromosomes reappear in their original number and form when the male pronucleus develops in the egg, and in cases where the inner constitution of any chromosomes is known, it always exhibits the same series of chromomeres in the stages where they are manifest. The most convincing case of this is the one described by Wenrich (1916) for the grasshopper *Phrynotettix magnus* (Fig. 1). In this animal certain chromosomes are individually recognizable by characteristics of size, form, and behavior. By a careful detailed study of these during spermatogenesis, Wenrich was able to show that each is made up of a definite number of chromomeres and that these are individually different in size and position within the chromosomes. One such chromosome was traced in thirteen different individuals, and showed great constancy in its internal organization (Fig. 2). When it is remembered that this precision of structure appears in these minute particles of matter after they have passed through all the violent treatment inherent in microscopical technique, it is remarkable to find the degree of exact-

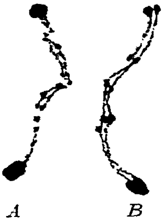


FIG. 1

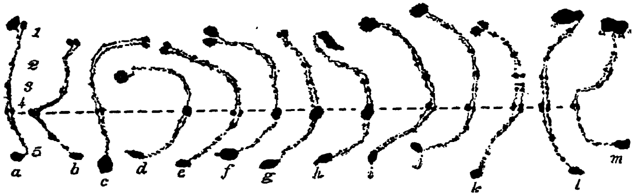


FIG. 2

FIGS. 1 and 2: FIG. 1.—Two examples of the same chromosome "B" from *Phrynotettix* from different first spermatocytes showing correspondence in number, size, and position of the chromomeres (Wenrich). ×3000. FIG. 2.—The same chromosome "B" shown in Fig. 1 but taken from thirteen individuals. A dotted line has been drawn between a series of homologous granules. The remaining, more conspicuous, chromomeres can be similarly compared (Wenrich). ×2200.

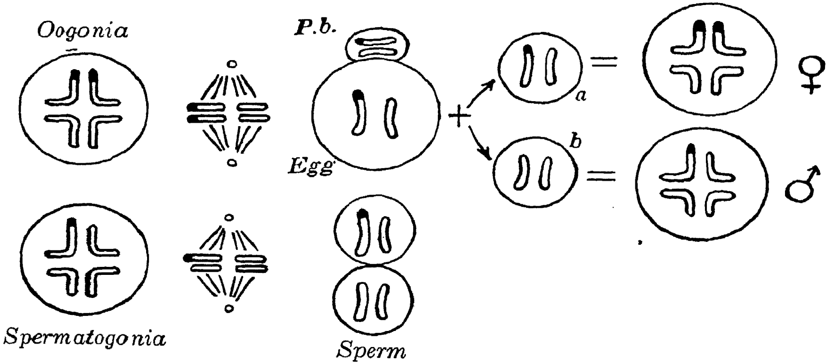


FIG. 3.—Diagrammatic representation of the position and behavior of the sex component in the chromosomes of *Ascaris megalocephala*. The black portion represents this differentiated part (Boveri).

ness which manifests itself. In addition to the well-marked chromomeres of sufficient size to be individually recognized there is some ocular evidence that there are still smaller subdivisions of the chromatin. With all these visible indications of structural differentiation, it is not going far to assume that these continue beyond the visible into molecular ranges.

Evidence as to functional differentiation must of necessity be indirect. So far it has not been possible to link up any particular chromomere with the manifestation of definite characters, although in *Ascaris megalocephala*, a small region of one chromosome, comparable to a large chromomere, was shown by Boveri to be the sex-determining material (Fig. 3). All the evidence for differential functions of aggregates of chromomeres is indirect proof of the specificity of their parts. It is to be noted also that Bridges has shown (1917) the causal relation between fragments of chromosomes and particular characters. Other evidence of functional differences associated with structural arrangements of the chromatin will appear elsewhere in this discussion.

V. THE DIFFERENTIATED CHROMATIN PARTS ARE DEFINITELY AND CONSTANTLY ARRANGED WITH REGARD TO EACH OTHER—FORMING A LINEAR SERIES

Since the microcosm of the cell represents an ordered state of materials, it is to be expected there will be, in all its parts, evidence of order. Particularly would this be true of the chromatin with its power of control over the system of which it is a part. Just what the nature of this order might be would not be evident a priori. What it actually is appears, however, with great definiteness upon a study of the nucleus when the chromatin is fully extended. Here the linear order of the chromomeres is beyond question. Many cells show this condition during the prophase of mitosis, but it is most marked in the germ cells just before the maturation divisions. Especially is this found in the Orthoptera, and in the case of *Phrynotettix*, already referred to, Wenrich not only demonstrated the linear order of the chromomeres but also the further fact that this expresses itself in a definite seriation. Linearity is the expression of a system, another and equally important feature of which is a fixed succession of elements. To these may be added also the element of distance between the chromomeres, which appears to be constant for any two, but variable throughout the series.

VI. THIS LINEAR ORDER IS NOT CONTINUOUS BUT IS SEGMENTAL, AND THESE SEGMENTS INDICATE, TO A CERTAIN DEGREE, THE INNER ORGANIZATION OF THE CHROMATIN

The appearance within the cell of a definite series of chromatin segments, or chromosomes, is one of its most obvious and striking characteristics. As fuller and more critical studies are made in well-defined groups of organisms, the precision of this numerical series becomes more manifest. Apparent variations find explanations based upon observed conditions within the chromosomes. Upon the assumption that the chromatin is the "idioplasm" and that it is made up of unit structures, associated with the development of body characters, it would naturally follow that this inner constitution should show evidences of its presence in the integration of higher units. The chromomeres probably represent the next higher order of association, but because of their minute character they have not been extensively studied. Their linear order is, however, very apparent. The chromosomes, on the other hand, because of their size and striking character, have been subjected to detailed analysis. From such studies the present conception of chromatin organization is largely derived. Since this is now causally linked to the organization of the body it is of the utmost importance to survey the evidence which is advanced in favor of the conception of a differentiated structure of the chromatin. As evidence of chromatin organization, finding expression through chromosome characters, the criteria employed are number, size, form, structure, and behavior.

1. *Chromosome numbers:*

It is, of course, entirely conceivable that there might be a congeries of chromatin units, acting as genes, the members of which could exist separately within the nucleus or associate themselves by chance. It is indeed possible that this may be the nature of chromatin organization in very primitive organisms. Within the higher forms of plants and animals, however, everything speaks for precision of association between the observable units of structure. This being true, it is to be expected that the manner and degree of association will be expressed by the external forms thus produced. Therefore, all indications of order and system in the chromosomes speak for like degrees of precision in internal structure. The conception of such an order in the nature of the chromatin units is summed up in the designation, "theory of the individuality of the chromosome."

Inherent in the nature of individuality is persistent unity. An individual, according to the definition of Huxley, is "a single thing of a given kind." In living things, along with this persistent and discrete unity, individuals commonly have the power to continue their kind by reproduction. When, therefore, we speak of the individuality of chromosomes we mean that each such structure exhibits these properties of individuals. Since all the cells of an organism come from the fertilized ovum they should all possess the same series of chromosomes, and, if a species is truly defined as a group of organisms directly related by descent, a like uniformity of chromosomes constitution might here obtain. Similarly, if larger groups represent the true expression of genetic relationships, there should appear evidences of this in the composition of the chromosome complex throughout the group.

Such a conception of the nature of chromosomes entirely excludes the possibility of chance associations of materials to produce them. There is none to deny the objective existence of these bodies as they take their place in the metaphase plate of the mitotic figure, and that they there reproduce themselves by separating into equivalent halves is not often doubted. There are those, however, who discount all the implications of the ordered events of the mitotic process, and deny the conception of chromosome individuality because the physical state of the chromosome changes during the period of intussusception and growth between cell divisions. Because these bodies do not maintain a persistent form but diffuse their substance for increase of surface during metabolism, thus confusing their outlines, certain types of minds conceive them to be temporary and incidental manifestations of cellular activity. That they should appear with regularity in successive generations is, to such, due to some mysterious integrating action of the "cell as a whole" or to a purely chance physical formation of fluid crystals out of an appropriate solution. It is not in the nature of the case that proofs of chromosome individuality supplied by unchanging form can be furnished, but for those who find no difficulty in conceiving the individuality of an organism through its manifold transformations of development, adequate proof of chromosome individuality is at hand.

Of first importance in this respect is the constant appearance in all the cells of the individual, and throughout the group of which it is a member, of recognizable chromosomes. As will appear later, it is sometimes possible to recognize particular chromosomes, but the means for such identifications are often lacking. In the absence of such unit identifications, we may have recourse to evidences of group constancy. The argument for this would be in these terms: Since all the chromatin is contained in the chromosomes and since in all the cells of an organism this chromatin appears in a like series of units, we assume that the members of this series are individually comparable. When it is recalled that these are all demonstrably derived from the original series in the fertilized ovum, it is really stating a fact to say that the chromosome complexes are individually comparable. When the cells of two organisms are compared and again a like series of chromosomes appears, there is some element of assumption involved if they are declared individually alike. Even here, however, there is little uncertainty when the individuals are the offspring of the same parents, for the source of the common group of chromatin units is the same. If comparison is made between parents and progeny and likeness again obtains, the analysis has been established phylogenetically. Such a genetical analysis was made by Carothers (1921) upon a short-horned orthopteran, *Circotettix verruculatus*, in which case it was found that three identifiable pairs of chromosomes, the members of which differ morphologically in the two parents, appear in the offspring in the sizes and forms which they possessed in the parents and in such groupings as chance segregation and recombination would produce. Here particular chromosomes could be traced back to the parents and homologized thus in the offspring. When now a large number of individuals of a species are studied and the same numerical uniformity prevails, there is every reason to believe that the chromosome group is the same. This feeling is greatly strengthened when larger and more inclusive groups like the genus and family show a like numerical constancy of their chromosomes.

The most striking case of this marked uniformity of the chromosome complex is shown in extensive studies upon the short-horned grasshoppers by McClung and his students. This family in North America consists of about 100 genera, including 800 species, and most of these have now been studied. It thus appears that throughout the group the somatic cells of the male contain twenty-three chromosomes while those of the female have twenty-four. Correspondingly, in the germ cells the diploid numbers are twenty-three and twenty-four and the haploid eleven or twelve in the male and always twelve in the female. The significance of these figures cannot be overestimated, for nothing but the most extreme precision of organization could preserve this common series of chromosomes through the millions of years in which this group of animals has existed, and in the innumerable multitudes of cells composing them. There is absolutely nothing to suggest the intrusion of chance into this incomprehensible picture of organic constancy.

2. *Chromosome sizes:*

While there would be a good reason for homologizing the members of chromosome complexes upon numerical equivalence alone, such genetic relationships appear the more probable when it is found that the members constitute a series graded by size. The twelve pairs in the Acrididae range thus from the smallest to the largest, which may be ten times its dimensions. Since these extreme members stand out in most cases with considerable distinctness, it seems reasonable to consider them homologous wherever found, but, judged by size alone, the intermediate elements of the complex are not certainly identifiable. Indeed, it must be realized that the uncertainties in microscopical processes are inherently so great that positive identification of nearly similar-sized elements is impossible by their dimensions alone. This circumstance does not, however, lessen the value of size, taken in relation with numerical constancy, as a basis for the assumption of chromosome individuality.

3. *Chromosome forms:*

As a means for the recognition of different kinds of organisms and for a determination of their relationships, morphological features have played a preponderating rôle. External configuration has been regarded as an almost certain index of internal organization. So true is this that in taxonomic work often a most trivial external marking is held diagnostic of specific, generic, or even more general relationships. What is true of the organism seems true also of its determinative parts even more accurately than body characters in some cases. The essential forms of chromosomes are very definite, and may serve for taxonomic determination. An instance of this is the case of *Mermiria bivittata*, an orthopteran. McClung (1917) reported this species as divisible into two groups upon the basis of a difference in form of one chromosome

(Fig. 4). Later Rehn (1919), in a revision of the genus, recognized these groups as taxonomically distinct. The genus *Mecostethus* may be distinguished from all other Orthoptera by its uniform, slender, rod-shaped chromosomes in the first spermatocyte as compared with the varied shapes of most other genera (Fig. 5). In most of the short-horned grasshoppers, the chromosomes of the diploid series are approximately rodlike, but in the *Circotettix-Trimerotropis* group, certain

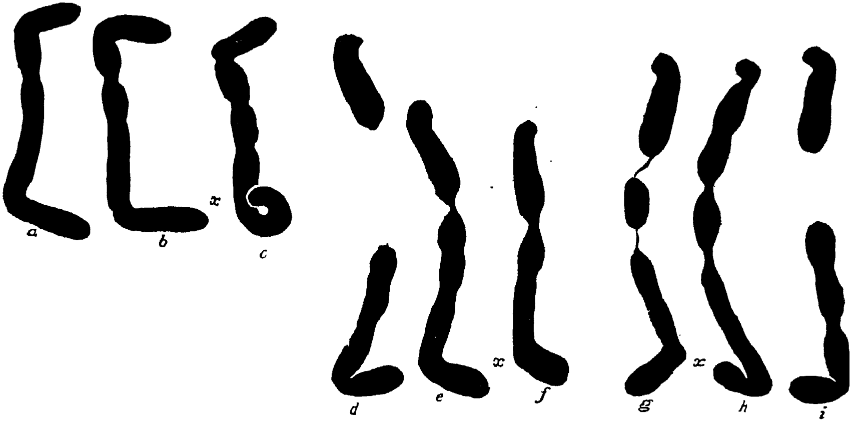


FIG. 4.—First spermatocyte chromosomes of *Mermiria*—hexad multiples showing the specific differences in the point of fiber attachment. *a, b, c* are from *M. maculipennis macclungi; d* to *i* are from *M. bivittata.* Structural differences, formerly overlooked, were observed when it was discovered that the chromosomes of the germ cells present these constant differences of form. *x*, accessory chromosome.

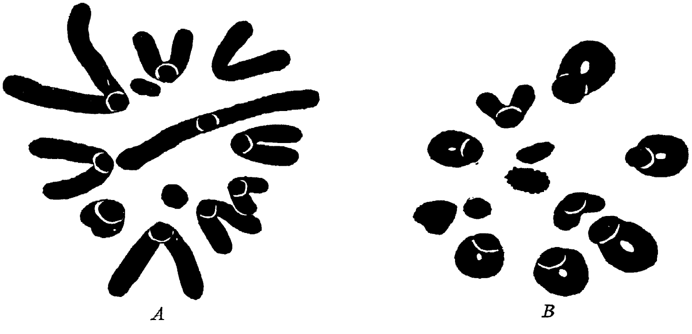


FIG. 5.—(*A*) *Mecostethus* chromosomes in the first spermatocyte metaphase. These tetrads are long, slender, and uniform in shape as compared with those of other genera of *Acrididae*, such as *Tropidolophus* (*B*).

ones may be J- or V-shaped, as shown by Carothers (1917), and by King (1924) (Fig. 6).

In addition to the characteristic general configurations of chromosomes there may be individual small structural differences that are highly constant. These show as constrictions in definite places, noted by Carothers and King, and as projections as reported by McClung (1914) for *Hippiscus* (Fig. 7).

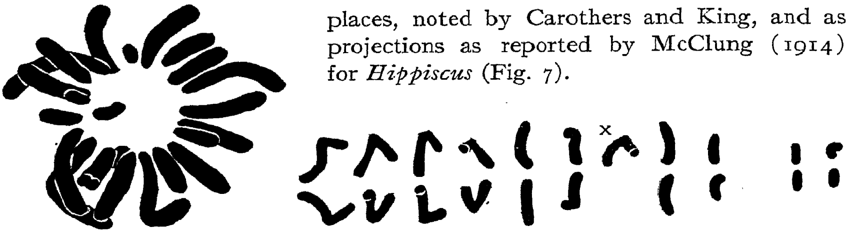


FIG. 6.—(*A*) Polar view of a spermatogonial metaphase of *Mermiria texana* in which all the chromosomes are of the rod type with the fibers attaching at the inner ends (McClung). (*B*) A spermatogonial complex of *Circotettix verruculatus* with its homologous elements arranged in pairs. Fiber attachment is at the angles of the bent chromosomes. The accessory chromosome *x* is unpaired (Carothers).

4. *Chromosome structure:*

The gross, external form of the chromosomes, as most clearly defined in the concentrated metaphase condition, is merely an expression of the inner, essential structure, which, at this time, is obscured by the dense massing of the chromatin parts. This internal differentiation of the chromosomes is most apparent in the extended state of the prophase where the chromomeres are widely separated. Here, as has already been mentioned, Wenrich was able, in certain cases, to trace from individual to individual the corresponding parts of chromosomes so small as almost to reach the limit of microscopic observation. There is every reason to believe that the definiteness of organization thus traced does not stop at the limit of our observational scope. An evidence of this fundamental organization is furnished by the relation of the chromosomes to the rest of the cell at the time of mitosis, as revealed by the point of fiber attachment. This seems to be a feature of great constancy, varying in only a few cases according to conditions not yet understood. The form of the chromosomes seems to be largely determined by the fiber attachment. In most orthopteran

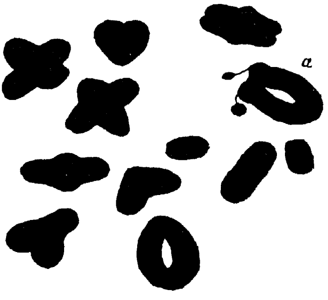


FIG. 7.—First spermatocyte chromosomes of *Hippiscus*. At *a*, a ring with two projections to which the archoplasmic fibers attach. This is a constant specific character (McClung).

species the chromosomes are rod-shaped in the diploid groups, the fiber attaching at the inner end in metaphase (Fig. 6*a*), but in others they are J- or V-shaped, in which cases they lie in the equatorial plate with the angle directed toward the axis of the spindle. At this angle, the fiber is annectant, and it varies along the length of the particular chromosome only very slightly from cell to cell, even in different individuals. Even when chromosomes join to form multiples, as in *Hesperotettix viridis*, the point of fiber attachment remains the same (Fig. 6*b*).

5. *Chromosome behavior:*

No evidence regarding the individuality of the chromosome is stronger perhaps than that furnished by the characteristic behavior of different chromosomes. Of outstanding significance here is the history of the accessory chromosome which has now so definitely been associated with the development of the differential features which distinguish the sexes apart. The whole course of this element during the maturation of the orthopteran male germ cells, where, unlike all the other chromosomes it is unpaired, is different from them, not in the exhibition of unique features, but in the rate and degree of the changes common to all chromosomes. Thus, during the spermatogonial divisions it is inclosed in a vesicle as are the others, but during the prophase this often becomes relatively enormous in size, showing a wide diffusion of the chromatin (Fig. 9). Again, at the time of the transformation of the final spermatogonial generation into the first spermatocyte, it condenses much more rapidly, and may be found as a somewhat homogeneous rod on the periphery of the nucleus while the other chromosomes are much diffused (Fig. 10). Like the euchromosomes, it completes a loop in the peritene stage, but within its own vesicle, and while remaining relatively condensed (Fig. 35). In the first maturation division it does not divide, and so goes into only half of the four spermatozoa which are formed from each first spermatocyte. It may be traced through every phase of the germ-cell generations without loss of visible

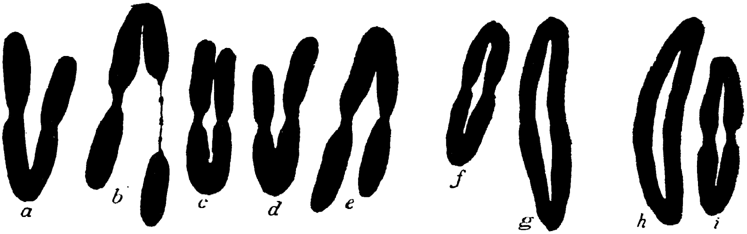


FIG. 8.—A series of euchromosome multiples, in *Hesperotettix viridis*, each involving two tetrads. Sometimes these are joined only at one end (*a* to *e*), in other cases at both ends (*f* to *i*). Fiber attachment remains constant, however (McClung).

identity, and always with marked peculiarities which serve to distinguish it from the other chromosomes of the complex.

The differential behavior of the accessory chromosome is most striking when it is united to another, forming a multiple. In the metaphase of the first spermatocyte under these conditions it is indistinguishable structurally from the euchromosome component, but in the telophase it remains fully condensed as in the metaphase, contrasting strongly, then, with the granular and diffused euchromosome element. This constitutes a striking proof of the individuality of the chromosomes, for the members of the multiple, although joined together into a unit of higher valence, still retain their distinctive properties.

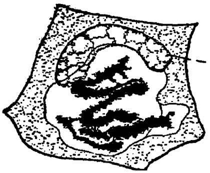


FIG. 9

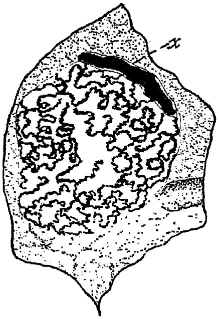


FIG. 10

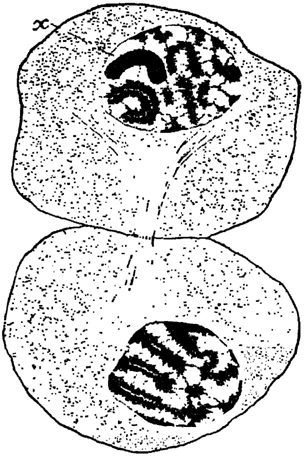


FIG. 11

FIGS. 9–11: FIG. 9.—Portion of a spermatogonium of *Brachystola*. The accessory chromosome x is contained in a separate vesicle (Sutton). FIG. 10.—A later spermatogonial stage in *Brachystola* with the accessory chromosome x condensed (Sutton). FIG. 11.—Telophase of first spermatocyte in *Brachystola* with the accessory chromosome x lying undivided in one cell (Sutton).

Of all the characteristic features of chromosome behavior, the most striking and significant is the orderly reproduction of each element during every mitotic division, with the exact preservation of all structural and functional characteristics. The fundamental significance of this act cannot be overestimated, because it furnishes the unquestionable explanation of the persistent individuality of each chromosome. This is the situation in all normal chromosome complexes, and it obtains also in cases where by any means chromosomes are added. Thus it sometimes happens that an additional or supernumerary chromosome is added to the normal complex, in which case the condition is perpetuated in all the resulting cells. Such cases have been reported by numerous investigators, and usually the condition is constant for all the cells of the individual, but Carroll (1920) has reported for *Camnula* variations between cells in different cysts of one testis. Here an additional dyad, or tetrad, may be present, and when found reproduces itself normally, behaving, when a dyad, like the accessory chromosomes, and normally, when a tetrad (Fig. 12).

In cases where the whole chromosome complex is duplicated by any means the double group maintains itself in normal fashion. So also does any multiple of the usual chromosome number. In general it may be said that whatever goes into a cell as its complement of chromosomes is maintained by individual reproduction of each member. One of the most striking instances of this is furnished by the work of Blakeslee and Belling on *Datura*. In this plant each of the twelve haploid chromosomes, in addition to being duplicated to produce the normal number, may have a third or fourth member present (Fig. 13). The whole group may come to have multiple representation, and in all instances the complex as established in the plant is maintained by individual chromosome reproduction (Fig. 14). The identity of the elements is indicated by the orderly association of homologues into multiple chromosomes with two, three, four, five, or six parts.

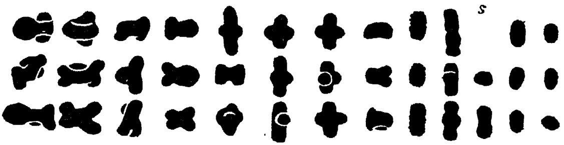


FIG. 12.—The first spermatocyte chromosomes from three cells of *Camnula* arranged with homologues in vertical columns. In the first row no supernumerary occurs; in the second row there is a supernumerary dyad *s;* and in the third row a supernumerary tetrad *s* (Carroll).

FIG. 13.—Chromosomes from *Datura*. The normal tetrad *a* may have an additional homologue, producing a hexad *b* (Belling and Blakeslee).

Similar cases of multiple elements and multiple complexes are not uncommon. McClung (1917) has reported multiple chromosomes in *Hesperotettix speciosus*, *H. pratensis*, *H. viridis*, *Mermiria bivitatta*, and *M. maculipennis macclungi*, but in these cases there are no additional elements in the complex, the compound nature of the chromosomes being due to more or less permanent associations between certain ones of the normal series, particularly involving the accessory chromosome. In *H. viridis* as many as six of the twelve first-spermatocyte chromosomes may be concerned in these combinations (Fig. 15), while other individuals of the same species have none. The important circumstance to note here is that whatever form of association is set up within the individual, it is maintained by reproduction in each chromosome involved. What are only occasional associations in *Hesperotettix* are permanent in *Chorthippus*, *Chloeältis*, and *Stenobothrus*. The form of combination and the particular members involved appear to be the same in all these cases, the largest two pairs unite end to end, forming a ring, as do the next largest two pairs, thus reducing the number of separate elements in the first spermatocyte from twelve to nine (Fig. 16). In *H. viridis* the accessory chromosome may combine with the largest free tetrad, making the separate elements of the complex only nine. Nothing is lost from the cell by these apparent numerical reductions—each chromosome maintains its identity and organization even to the finest observable details.

It is of the greatest importance for the conception of chromosome individuality to find that in these different genera, from different subfamilies, the same tendency to form multiples obtains and between the same members of the series, proceeding, in the instances thus far studied, from the largest elements down through the series, and producing the same structural forms. The details of the association processes are shown in various individuals of *H. viridis* where it is seen that the first step in the union of the non-homologous tetrads may be fusion at one end only, producing a V-shaped structure, which upon division in the first spermatocyte produces, in the resulting second spermatocytes, different chromosome numbers (Figs. 8, 15). In another individual, fusion may be complete at both ends of the two tetrads involved when a ring is

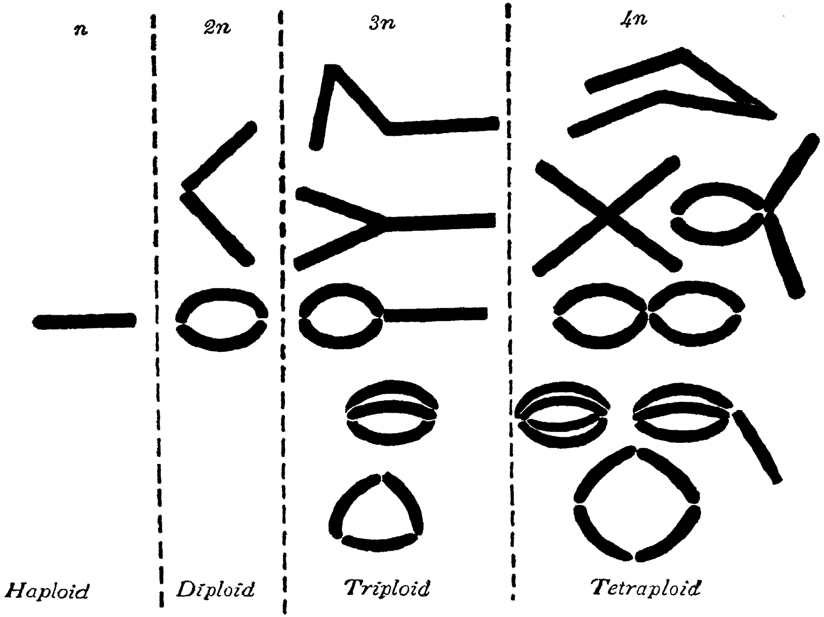


FIG. 14.—Diagrammatic representation of various chromosome multiples in the first spermatocytes of *Datura* (after Belling and Blakeslee).

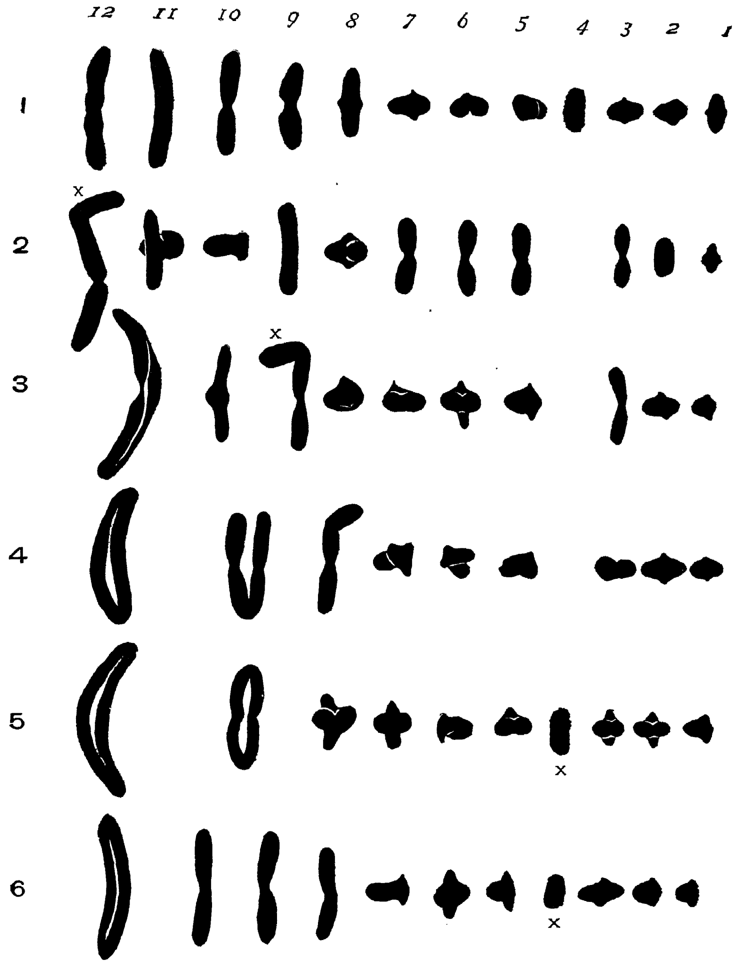


FIG. 15.—This figure illustrates the various arrangements of chromosomes found in different individuals of *Hesperotettix viridis*. In row 2, column 12, the accessory chromosome x is joined to the largest tetrad to form a hexad. In row 3, columns 12–11, the largest two tetrads unite into a ring-shaped octad, while in column 9 the accessory chromosome and a tetrad form a hexad. Row 4, column 12–11, exhibits a ring octad; column 10–9, a V-shaped octad; and column 8, a hexad, involving the accessory. All the cells of an individual have the same combinations (McClung).

formed, whose subsequent division does not result in numerical chromosome variation in daughter-cells. That the temporary association of chromosomes in *H. viridis* represents, in principle, the form of combination permanently established in *Chorthippus* and *Chloeältis*, was earlier ably argued by Robertson (1916) from numerical relations and structural features. Woolsey (1915), working with Robertson, found in *Jamaicana*, a locustid, conditions similar to those in *H. viridis*.

Multiple complexes, where each element is repeated two or more times, are commonly the result of accident or experimental disturbances. Failure of the cell to divide after the chromosomes have separated in mitosis results in a "giant cell" which contains double the chromosome number. In subsequent cell divisions all the chromosomes reproduce themselves as usual. Hartman (1913) has described these familiar conditions in the Orthoptera, and they occur in many animals. In plants the history of multiple complex cells has been traced through genetical results more fully than in animals. The well-known case of *Oenothera gigas* with its double normal complement of chromosomes is typical of many plants. The case of *Datura* has already been mentioned.

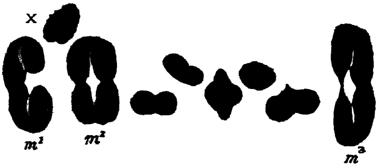


FIG. 16.—Lateral view of the first spermatocyte metaphase chromosomes of *Chloealtis* with three ring-shaped octads m^1, m^2, m^3 (McClung).

There are a number of instances among plants where there are a series of varieties, or species, differing concomitantly in chromosome numbers, which are often multiples of the lowest number in each case. Thus Tahara (1921) finds that various species of chrysanthemum show haploid numbers of nine, eighteen, thirty-six, or forty-five, and that in many cases, even in the maturation stages, the chromosomes reproduce themselves normally. Similarly, Blackburn and Harrison (1921) state that the fundamental haploid number of seven in *Rosa* may appear in different species as fourteen, twenty-one, twenty-eight, thirty-five, or forty-two in normal number. Sax (1922) reports that in the einkorn group of wheats the haploid number is seven, in the emmer group fourteen, and in the vulgare twenty-one. Normally these chromosome groups maintain themselves throughout the life-history of the plant.

The instances of multiple complexes already given have been noted first in the germ cells, and thus are perpetuated in the resulting body cells. It appears, however, that they may arise during the course of development and then be confined to certain tissues or organs. One of the most interesting cases of this character was reported by Holt (1917) in the pupal cells of *Culex*. Here it appears that during the complicated changes of histolysis and histogenesis the chromosomes in the gut cells undergo repeated longitudinal divisions so that instead of the normal six there may be as many as seventy-two present in a cell. Without further facts from which to gauge it might be assumed that these conditions in *Culex* constitute a distinct exception to the rule of orderly chromosome reproduction with the consequent preservation of chromosome individuality. Although these unusual conditions occur in cells which later break down into material for the reconstitution of the gut epithelium of the adult insect, and hence might not be of significance, even under these circumstances no essential alteration in normal relations and behavior of the chromosomes occurs. Each chromosome present shows the characteristics of one of the three kinds of the haploid group, and participates with the others of its sort, no matter how numerous they may be, in forming chromosome vesicles in the prophase of mitosis and in other cellular changes. Although the amount of chromatin is greatly increased, without consequent proportionate enlargement of the cell, nothing new in kind is added, and chromosome relations, other than numerical, are not altered. Whether there are six, nine, twelve, fifteen, or seventy-two chromosomes in a cell means nothing more, so far as chromosome individuality is concerned, than that there are present two, three, four, five, or twenty-four representatives of each haploid form.

Attention has already been called to the significance of apparent variation in chromosome numbers for the theory of chromosome individuality. In every case such seeming differences require careful study to determine just what behavior of the chromosome is involved, and are not to be accepted as prima facie evidences of chromosome inconstancy. The classical case of chromosome diminution in *Ascaris*, as reported by Boveri, presents a picture of complete constancy in the behavior of germ-cell chromosomes, accompanied by an altered organization in those of somatic cells. Two things would seem true in this case: first, the chromosomes of the germ cells are units of higher order—multiples; and second, certain materials are not required in the development of the body of *Ascaris* in its present form. Although these conditions are different from what is normally found in development, it is to be noted that there is no internal element of inconstancy involved, but only a different order, the terms of which are not understood.

Hance (1917) has made a careful metrical study of the chromosomes in pig embryos, where the somatic cells show separate chromatic bodies varying in number from forty (the normal chromosome number in the germ cells) to fifty-eight. The significant finding in this case is that there is no addition or loss in chromatic material, but merely an altered integration. The longer members of the complex are subject to limited fragmentation, and the resulting parts behave as entire chromosomes. When these enlarged complexes are compared with the normal ones of the germ cells it is found that by adding certain proportionately small elements to the ends of larger members, the size and numerical relations can be harmonized. The same author (1918) found similar conditions in *Oenothera scintillans*. In this plant the normal chromosome number of fifteen may, by fragmentation, be raised to as high as twenty-one. Again numerical and size seriation may be restored in any cell with unusual chromosome number by uniting the fragments to the proper members of the series. In all these cases it is important to note that the separate chromatin units, no matter what their origin or valence, reproduce themselves. This is only another way of saying that the ultimate chromatin units perpetuate themselves individually, since the total of the chromatin aggregates represent the sum of the ultimate chromatin units.

Apparently the supreme test for the differential nature of the chromosomes is furnished by the processes of maturation. Even in normal reproduction this is a trying period, but in hybridizations disharmonies are strikingly revealed and usually result in partial or complete sterility, due to the failure to complete germ-cell formation. It is interesting, therefore, to note the behavior of the chromosomes in plant hybrids which produce fertile offspring. If species with the same chromosome numbers are crossed, the chromosome behavior is essentially normal. If the parents have different numbers, tetrads are formed up to the limit imposed by the lower number. The remaining elements, being without homologues, do not synapse, and divide in only one maturation division. It is not without significance here that these unpaired elements behave in division much like the single accessory chromosome, dividing sometimes on the first (14+21) combinations in wheats, according to Sax, sometimes in the second (7+14). These facts indicate the essential unity of the chromosome organization which shows itself relatively independent of the external conditions. Blackburn and Harrison observe that in rose hybrids the unpaired chromosomes are gradually eliminated from the germ line, so that eventually the number becomes that of the parent with the lower numerical series. Because this elimination does not always follow the same course there may thus be produced stable races with the same chromosome numbers but with different somatic constitution.

Since the essential behavior of all chromosomes must be the same, individual peculiarities are confined to differences in the comparative rate, time, or degree of a common series of changes. The accessory chromosome is very marked in respect to these differential characters. There are, however, other instances of similar conditions among the euchromosomes of which the activities of the "selected chromosomes" in *Phrynotettix* as described by Wenrich (1916) are of great importance. In general, these three of the haploid series of elements are marked by a more intensive staining reaction in the first spermatocyte prophase which apparently betrays an early and strong concentration of the chromatin. Because of this fact these chromosomes may individually be identified at a time when, it is often asserted, all traces of chromosomes as such are lost (Fig. 17).

Each is different in particulars of structure and behavior, and may thus be distinguished from the other members of the complex in any individual of the species examined. Some features in the behavior of these elements are of great interest and significance. The chromosome, indicated as "B" by Wenrich, in eleven out of thirteen animals has one homologue smaller by the absence of a terminal chromomere. At the time of the first spermatocyte division this element divides so that each second spermatocyte receives a half of the larger and of the smaller component, thus clearly showing the division to be equational (Fig. 17). Chromosome "C" is also heteromorphic, but divides in the first spermatocyte in half of the cases equationally and in the other half reductionally (Fig. 17). Thus, in one of the most significant activities in the history of the chromosomes, these elements manifest their individuality so strongly that they can be recognized from cell to cell and from organism to organism.

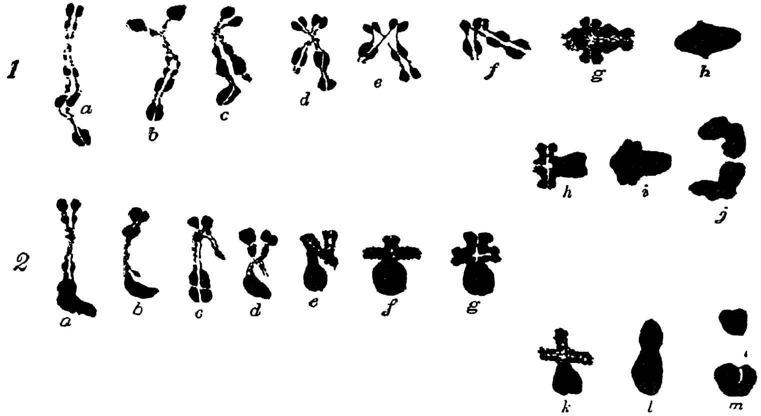


FIG. 17.—Differential chromosomes of *Phrynotettix* in late prophase of first spermatocyte. Row 1 shows successive stages in the condensation of Wenrich's chromosome "B" *a–h*. The terminal granule is missing in one homologue so that the tetrad is unequal. Row 2 represents the structure and behavior of chromosome "C" which is also unequal, but which may divide equationally *h–j*, or reductionally *k–m*.

Aside from forming a basis for the recognition of individual elements the behavior of chromosomes "B" and "C" clearly demonstrates that the formal and elaborate theories of prereduction and postreduction, which have been provocative of so much useless discussion, are of no significance. The two maturation divisions constitute a unit process, serving to assort by chance the four elements of any tetrad in relation to the elements of all other members of the complex, and the segregation of homologues may occur in the first or second.

It thus appears that, judged by criteria of number, size, form, and behavior, the individual segments of the chromatin series indicate the differential character of the inner organization of the chromatin. That is to say, the chromosomes not only differ between themselves, but individually they possess a characteristic structure. Taken together the chromosomes represent the sum total of all the elements of control over the processes of metabolism, irritability, contractility, reproduction, etc., that are involved in the life of an organism, but in the measure that organisms differ, so do the natures of their controlling mechanisms. These differences must extend over to the ultimate units of structure and the chromosomes, as aggregates of these, are at once an expression of the underlying unity of vital processes in all living things and an index of their specific and individual variations.

VII. THESE SEGMENTS—THE CHROMOSOMES—PERPETUATE THEMSELVES WITH EXACT PRESERVATION OF THEIR INNER STRUCTURE BY A PROCESS OF DUPLICATING EACH ULTIMATE UNIT BY GROWTH THROUGH INTUSSUSCEPTION, WITH A SUBSEQUENT DISTRIBUTION OF THE TWO PARTS INTO SISTER CELLS

If the chromosomes are such definite and characteristic structures as the theory of their function demands, there must exist a process of incredible exactness for their perpetuation. Groups of organisms whose kind have been in existence for millions of years present today, in every cell of every individual, exactly the same visible complement of chromosomes. During this time there have been sequences of changes in inorganic matter so extensive as to produce whole series of chemical elements from one, and yet through all the manifold and apparently unstable conditions of organic existence the chromosomes of known systematic groups present an appearance of fixity and stability that is marvelous. The intimate processes by which this constancy is maintained remain as yet a mystery, but the mechanism of chromosome reproduction is fairly apparent in each cell division. Indeed it was this picture of precision and unity in the mitotic process which led Roux and Weismann to the conception of the chromosome theory of inheritance. Assuming that there is a division of labor among the cell parts and that one of these is regulatory or directive of ordered cell processes, the implications of mitosis are almost compelling in their significance.

A review of the process of mitosis may serve to emphasize the significant aspects in relation to the hereditary mechanism. A newly formed cell is, in most cases, one of two nearly equal cells. It contains, part by part, half of the parent unit, but each of these is half the original size. Before another division can occur, some approximate restoration to normal proportions of all portions of the cell must take place. Material from the outside must be brought in, and there made over into the likeness of each of the many ultimate structural units—all of which is a part of the as yet unexplained processes of metabolism.

So far as the chromatin is concerned, it is observed that the original dimensions of each chromosome have been restored, and when they are fully extended in the spireme it is obvious that all visible chromomeres are of the proportions which characterized them in the mother-cell. This is the necessary conclusion which must be drawn from the conditions observed in a series of dividing cells in any established tissue. In the process of egg cleavage where differentiation is occurring and the cells are merely dividing existing material, actual dimensions are quickly reduced, although in the chromatin relative proportions appear to be retained.

When the time for another mitosis comes in the cell, the significant feature of the process clearly lies in the behavior of the chromatin. While in the growth changes it has disposed itself upon the nuclear membrane so as to offer the greatest possible surface at this osmotic barrier, it now becomes extended in a linear manner so as to reduce the lateral dimensions of each constituent part almost to the limit. When thus drawn out the individual chromomeres divide lengthwise into equivalent parts, so that the single extended thread, representing the chromosome, becomes a double one, and, we say, the chromosome has split lengthwise. Judging the significance of the result by the nicety of the process we must conclude that something over and above the mere exactness of mass division is here involved. This could be attained much easier. It is merely a matter of observation that each visible part of the chromosome, down to the limit of our observation, has accurately reproduced itself, and, at the same time, has preserved its relations to the other members of the series. In other words, the organization of the chromosome in its entirety has been maintained and duplicated. This is the essential feature of mitosis, so far as it involves the chromatin—all the subsequent steps being connected with details of separating the equivalent halves of each chromosome and of distributing them to two cells.

Following on this extreme elongation of the chromosomes and their lengthwise division, there comes a reverse process of concentration. The chromosomes become shorter and shorter, the chromomeres run together, and the formerly granular-appearing structure of the fixed and stained preparation is sharp in outline and apparently homogeneous in structure. Very much the same appearances may be observed in living cells under favorable conditions. On the attainment of the final stages of chromosome concentration the nuclear membrane disappears, and the chromosomes form a flat plate in the equator of the cell.

For convenience of discussion it is desirable to consider the parts of the cell separately, especially when, as in the case of the chromosomes, they are of primary significance in function. It must always be remembered, however, that the cell is the functional unit and that the behavior of any element has to be regarded strictly in relation to the complex of which it is a portion. Thus, in the process of mitosis, the complicated changes of the chromosomes during the prophase have accompanying and related changes in the cytosome as a necessary concomitant. These show themselves particularly in the specialized portion of the cytoplasm called the archoplasm. At first all that may be seen of this is one, or sometimes several, centers on or near the nuclear membrane from which distinct radiations proceed. Soon it is noticed that the radiations, or astral rays, converge upon a central granule, or centrosome, which very early divides into two. These move apart upon the nuclear membrane, each forming the center of a system of radiating fibers of increasing length and number, and connected together by a spindle-like bundle of fibers. By the time the chromosomes have become fully condensed, the centrosomes have reached positions at opposite sides of the nuclear area, the cytoplasm has largely become organized into astral or spindle fibers, and the nuclear membrane, as such, has disappeared. The result is a definite bipolar condition of the cell with the chromosomes suspended in the equator and connected, each by a fiber on either side, to the centrosomes.

In the metaphase thus established, distinction between nucleus and cytosome, as areas separated by an osmotic membrane, is lost, although the materials of each occupy much the same relative positions. The so-called fibers forming the archoplasmic apparatus are not visible as such in the living cell, but the spindle-shaped mass which they form about the chromosomes may be driven entire through the cell by centrifugal force, showing that this material is of greater density than that about it, as it definitely appears to be in fixed material. Moreover, there is something of great definiteness about the fibers which run from the chromosomes to the centrosomes. For any one chromosome they have very well-defined and .constant points of attachment, and clearly mark positions in the chromosome where its relation to the archoplasm is different and more intimate than elsewhere. This condition is at once an evidence of precision and definiteness in the organization of the chromosomes and of constancy in relation between the parts of the cell. If the archoplasmic apparatus were only a means for separating the parts of the dividing chromosome, attachment of contracting fibers at any point would be sufficient, but the conditions as they exist are, on the contrary, indicative of fundamental features of cellular organization.

Following upon the arrangement of cell materials into the balanced state of the metaphase bipolar figure, there comes a disruption of the previously existing unity of the cell which shows itself in a rapid movement of the chromosomes from the equatorial plate to the regions of the two centrosomes. These anaphase movements are equal in time and degree on the two sides of the equatorial region, and involve not only the chromosomes but the other portions of the cell also. The result is to transform the bipolar condition of the metaphase with all the chromosomes and much of the archoplasm concentrated in the equatorial region of the cell into the bipolar state of the anaphase where the concentration of chromosomes and archoplasmic substance is at the poles, leaving a lighter and disorganized plane in the equator. Along with this polar movement, the chromosomes undergo a loosening up of their structure which separates the denser from the more fluid substances.

Limiting the anaphase to the period marked by the movement of the daughter-chromosomes from the equatorial plate to the poles, the telophase completes the changes necessary to produce two cells in the resting stage. Essentially these consist in further development of fluid materials about the chromosomes with the production of a distinct spherical nucleus and the separation of the original cell body into two with a rearrangement of the highly polarized condition into a more concentric one. On the completion of the process of mitosis, therefore, there are two cells containing the materials of the mother-cell, but so divided as to duplicate its organization, at least in the chromatin, to the finest observable details.

VIII. IN NORMAL BIPARENTAL REPRODUCTION THE CHROMOSOMES APPEAR IN THE ZYGOTE AS A DUPLICATE SERIES, THE MEMBERS OF WHICH ARE DERIVED EQUALLY FROM THE MALE AND FEMALE PARENTS

So far as genetical evidence indicates, the rôles of the two parents are essentially the same—inheritance of any character or group of characters may show the peculiar stamp of either the maternal or paternal parent in equal degree. On the assumption that inheritance is the manifestation within a continuous series of organisms of the same spatial and time relations of given materials, then its mechanism must be material and parallel in the two parents. That is to say, the single cell, the fertilized ovum, which constitutes the first stage in the development of a new individual, must have received, among the materials contributed by the two parents, equal and similar groupings of some of these. It is very obvious that, in most instances, the mature egg and sperm are extremely unlike in size, and that, so far as material contribution is concerned, the female parent is much the larger donor. But, as is indicated at length elsewhere in relation to fertilization, an inspection of the fertilized egg shows that an almost exact equivalence in respect to the chromosomes contributed by the two parents obtains (Fig. 18). This was first definitely established by Van Beneden in his remarkable paper of 1883, and its significance cannot be overestimated.

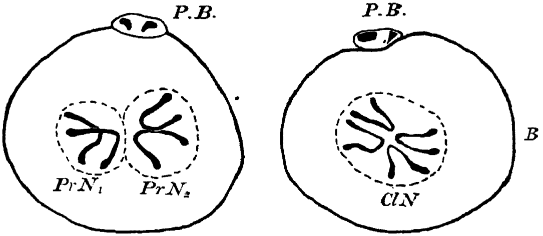


FIG. 18.—Two late stages in the fertilization of *Ascaris megalocephala* variety *bivalens*. In *A* the pronuclei (PrN_1 and PrN_2), each showing two chromosomes, are in contact. Above, *P.B.*, is the second polar body with two univalent chromosomes. Later (*B*) the pronuclei fuse into the cleavage nucleus which contains the two chromosomes of the egg pronucleus, plus the two from the sperm (Van Beneden).

Fertilization follows the occurrence of an almost identical process of preparation in the ovum and sperm, and cannot well be considered apart from the implications of this process. While through these preparatory changes in the egg and sperm, each approaches their union with half a complement of chromosomes, but with this practically identical in the two cases, it appears that so far as the chromosomes are involved, fertilization is a means of providing the newly formed individual with a double chromosome series. Since parthenogenesis and various experimental data show that either series is all sufficient in determining development, it follows that the normal organism is essentially duplex in its hereditary constitution and that every character represents a resultant of the interaction or, as it sometimes appears, antagonistic action of paternal and maternal influences.

The cytological facts of fertilization are well established from many sources. In the variety *bivalens* of *Ascaris megalocephala* two chromosomes are found in the egg pronucleus and two in the sperm pronucleus. The variety *univalens*, instead of two chromosomes in each pronucleus, shows only one, but the parental contributions are the same. When, however, these varieties cross, one gamete contributes two chromosomes, the other only one. Definite numerical relations of this character have been found in large numbers of cases. The conditions thus established in the zygote are continued on into all the resultant cells, both somatic and germ, in nearly all organisms (Fig. 19). *Ascaris*, in this respect, presents a very exceptional case, because the chromosomes in the body cells fragment and cast out a large proportion of their substance, while in the germ-cell line they continue unchanged (Fig. 20). There is here, however, no difference in the behavior of the parental contributions, the two series being equally fragmented.

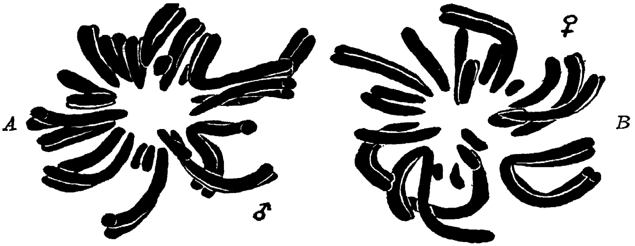


Fig. 19.—A comparison of the chromosomes in *A*, a spermatogonial complex, with those of *B*, from the follicle cell of a female *Mermiria maculipennis macclungi*, shows the same seriation in size and shape. The female complex, however, has an accessory chromosome as a part of each V-shaped element, while in the male only one of these is of that composition (McClung).

When, as in the case of *Ascaris megalocephala* var. *univalens*, there is but a single chromosome in the mature gametes, it is entirely obvious that they are homologous and that of the two in the zygote one is paternal, the other maternal in origin, but in instances where there are large numbers, aside from numerical equality, it is often difficult, and sometimes impossible, to establish the individual correspondence between the parental chromosome contributions. There are, however, many cases where parallelism exists not only in numbers but in recognizable features of individual chromosomes; and where even one pair of a coherent complex are homologized, the presumption for similar relations in the others is raised to a high degree. If it can be granted for the purposes of the present discussion that numerical chromosome correspondence in the two gametes is indicative of equality in parental contribution, the detailed evidence for this will be presented in the consideration of changes undergone by the chromosome complexes of egg and sperm in preparation for fertilization.

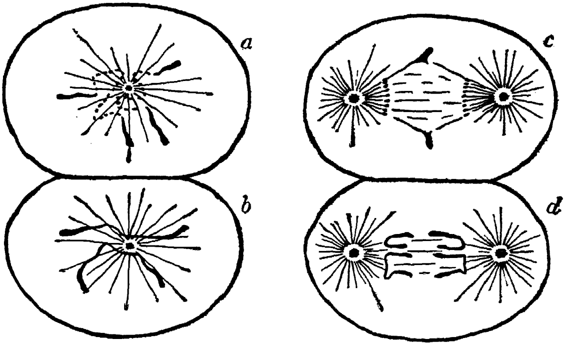


FIG. 20.—Differentiation of germ and body cells is shown in these figures of *Ascaris megalocephala* cleavages by Boveri. At *a* is a polar view of a metaphase plate in which the central portion of each chromosome is fragmented into a number of smaller elements. In the anaphase (*c*) these divide, while the large ends are left behind to disintegrate. Cell *b* shows no such fragmentation. It appears that *a* is the forerunner of the body cells and *b* of germ cells.

IX. IN PREPARATION FOR THE UNION OF THE SPERM AND OVUM IN FERTILIZATION, BY WHICH THE DUPLEX CHROMOSOME SERIES IS ESTABLISHED IN A NEW INDIVIDUAL, EACH OF THESE GERM CELLS REDUCES ITS OWN DUPLEX SERIES TO A SIMPLEX ONE BY SEGREGATING ITS MATERNAL AND PATERNAL MEMBERS OF EACH PAIR INTO DIFFERENT CELLS BY CHANCE DISTRIBUTION IN RELATION TO THE OTHERS

Of all the phenomena of living things, the process of maturation in the germ cells is perhaps the most suggestive. It is at once the last stage in the relation of the homologous chromosome pairs of the preceding generation and the preparatory stage of the following generation, while, in a large measure, independent of the one in which the changes take place. Aspects of the past, the present, and the future mingle in the activities of the chromosomes at this significant period, but the implications are clearly of the future. Maturation is a preparation for what is to come and, despite all the obvious physical differences in the germ cells produced by the two sexes, consists of parallel and almost identical steps in the developing egg and sperm. Even in parthenogenesis, where the sperm does not play its usual part, the egg undergoes the steps preparatory to union with the paternal cell, although its own polar body, thus produced, may be the nearest approach to the sperm's contribution.

The mechanism of maturation consists of two mitotic divisions rapidly succeeding each other without an intervening resting stage; the result is the production of four cells from one, each of which has half the original number of chromosomes. It is thus often stated that maturation is a device for reducing the chromosome numbers to one-half, so that, upon union of the two mature germ cells, the sperm and ovum, the normal number is restored. In the absence of such a reduction the chromosome number would be doubled upon each fertilization—an obviously impossible condition. While this result is certainly accomplished, much more than a mere numerical balance results from the maturation processes. Here occur the sorting and recombination of ancestral chromosome contributions which apparently constitute the distinctive feature of sexual reproduction. Certain very important internal changes, which will be considered later (XI, p. 651), occur in the chromosomes prior to these mitoses, but the maturation divisions accomplish the actual distribution of ancestral units into different functional or non-functional cells, and they will be discussed from this aspect.

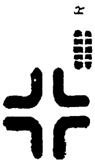
Viewed in this way, maturation is the opposite, or complementary, process of fertilization. When the zygote is formed, egg and sperm make equivalent chromosome contributions. Each individual chromosome of the ovum has added to it another of the same kind, recognizable by characteristics of size, form, and behavior, so that the new individual starts its development with a double series. This duplex condition is always maintained by the somatic cells, but is terminated in the germ line by the maturation divisions. At this time also, usually in the paternal, but sometimes in the maternal, line, a chromosome distinction between the sexes is anticipated. It is this process of chromosome numerical reduction and segregation which will now be considered (Fig. 21).

Maturation of the egg and sperm are essentially the same, so far as the chromosomes are concerned, the only difference being that in the egg only one of the four groups remains in a functioning cell, while in sperm formation all are potentially functional. Undoubtedly, this device for throwing out of possible operation certain chromosomes is of great significance in sexual reproduction but it is not of immediate concern in this consideration. Because maturation of the paternal cells, or spermatogenesis, lacks this added element of chromosome elimination found in oögenesis, it will be used as the basis of the description of maturation.

The simplest case for the understanding of numerical relations is one in which the zygote has received a single chromosome from each parent. Such a case is furnished by *Ascaris megalocephala* var. *univalens* (Fig. 19). At the time of germ-cell maturation these two elements are separated and distributed to different cells, thus reducing the number to one in each, and restoring the conditions to what they were in the gametes producing this zygote. In the variety *bivalens* of this *Ascaris* the zygote has four chromosomes, and at the time of maturation this is reduced to two. A genus of plants, *Crepis*, has species with three, four, and five pairs, the members of which are segregated in maturation as in *Ascaris*. In a similar manner in all organisms the members of each pair, no matter how many there may be, are separated from each other and left in different cells. The result for the whole chromosome complex is to reduce the diploid number of the zygote to a haploid condition so that one member of each pair is represented.

Commonly for each recognizable element there are two present, but in the males of many insects and other animals, and some plants, the cells show an uneven number and hence an unpaired chromosome. This element is always distinguishable by peculiarities of size and behavior, so that it may not be confused with the others. At the time of chromosome division in maturation, not having a mate from which to separate, it simply passes into one of the two cells formed at this time without change (Fig. 11). In some insects this odd chromosome may have a mate which, unlike the members of other pairs, is of smaller size or of different shape. In all cases, however, the female shows no irregularity in the pairing of its chromosomes. Where there is an odd chromosome in the male, it has a mate in the cells of the female, and where there is a diversity of size or form in the mate of this chromosome in the male, there is uniformity in the female. A study of the history of this odd, or X-chromosome shows that it alternates regularly between the male and female lines, while its mate, or Y-chromosome, if it have one, is restricted to the male line. It is apparent, therefore, that these elements are associated in some way with sex, but here it is necessary to note only the nature of their segregation.

During maturation the segregation of homologous chromosomes, which stands out so prominently in the process, is complicated by two circumstances. First, the homologues, previous to their separation, are most intimately joined together, and second, they are then longitudinally divided as in preparation for an ordinary mitosis. The result of this combined fusion and splitting is to produce, in the first spermatocyte or first oöcyte, a series of chromosomes one-half the normal in number and each composed of four parts or chromatids. The total number of these chromatids is equal to that found in any diploid complex at the time of a mitosis (Fig. 21). That is to say, this haploid complex has within it all, and only such, chromatids as are present normally in a cell upon division. The four chromatids of each tetrad chromosome are then, by the two maturation divisions, separated and distributed into four sperms in the male, or into the egg and its polar bodies in the female. Obviously, as regards the paternity of these four elements of each tetrad, two came from the male parent and two from the female. One of the maturation mitoses, then, operates to segregate the parental constituents while the other witnesses such a division

	TOTAL CHROMATIDS	NO. OF CELLS	FORM OF CHROMOSOMES	NO. OF CHROMOSOMES	CELL GENERATIONS
	46			23	Primordial germ cells
	46			23	Primary spermatogonia
	46			23	Secondary spermatogonia
Spermatogenesis	44+2	1		11+1	First spermatocyte
	2×11=22 2×12=24 46	2		11 or 12	Second spermatocyte
	11+11+12+12=46	4		11 or 12	Spermatids
					Spermatozoa
	In all cells of group	In group		In each cell	

GRAPHIC OUTLINE OF PATERNAL GERM CELL HISTORY

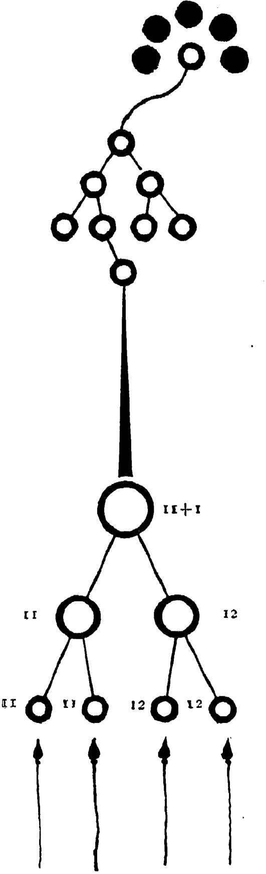
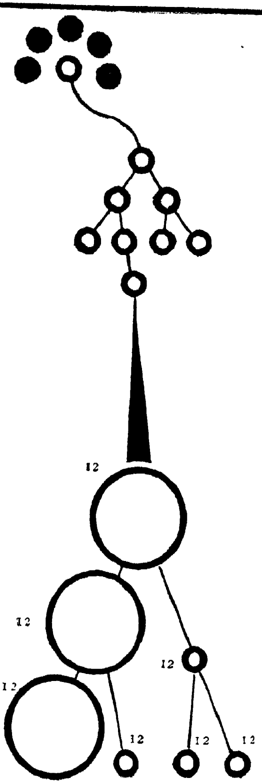
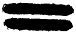
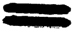
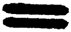
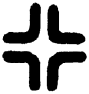
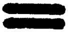
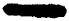


FIG. 21.—A diagrammatic comparison of the germ-cell histor

Graphic Outline of Maternal Germ Cell History	Cell Generations	No. of Chromosomes	Form of Chromosomes	No. of Cells	Total Chromatids
	Primordial germ cells	24			48
	Primary oögonia	24			48
	Secondary oögonia	24			48
	First oöcyte			1	48
		12			
	Second oöcyte	12		2	48
	Oötids = Ovum and polar bodies	12		4	48
		In each cell		In group	In all cells of group

Oögenesis

le and female, giving details regarding cell and chromosome behavior as commonly takes place in mitosis. Because of these facts one maturation mitosis is spoken of as a reduction or segregation division and the other an equation division. It was formerly thought that all chromosomes behave alike and that the first or second maturation, according to the interpretation of the investigator, is the reduction division in all plants or animals. In some cases where positive identification of the constituents of the elements is possible, however, it has been found that the same chromosomes may sometimes be reduced in the first division, and sometimes in the second, and also that not all the elements of the complex act alike in the same division (Fig. 17). Also, as will appear later, in preparation for the maturation divisions the parental homologues react upon each other so that when they separate they are no longer as they were when they were brought together in the zygote. When speaking of the segregation of parental contributions, therefore, it is now necessary to refer to the constituent parts of the chromosome. As was described elsewhere, these parts, or chromomeres, are individually different in size and position within the chromosome, and at one time in the history of the germ cells are so intimately associated that any four of these homologous levels or parts of a tetrad cannot be differentiated as to their origin.

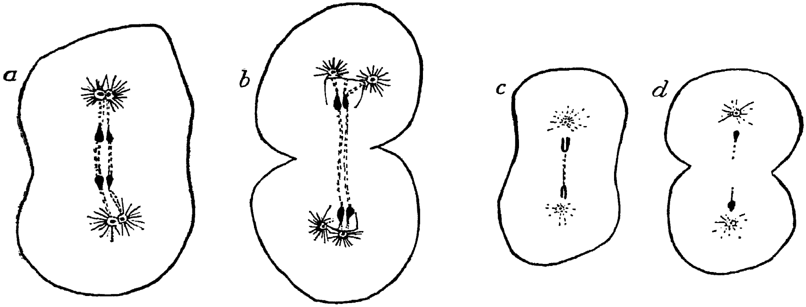


FIG. 22.—Stages in the spermatogenesis of *Ascaris megalocephala*, var. *univalens* (after Brauer). In the anaphases of the first spermatocyte, *a*, *b*, the two chromosomes are dividing. Corresponding stages of the second spermatocyte, *c*, *d*, show the reduction of the chromosome to one in each spermatid.

With regard to the distribution of the parental constituents of the chromosome complex into the four sperms derived from each first spermatocyte there are two possibilities: first, the paternal elements of the zygote may be separated *en bloc* from the maternal and placed in different cells; or second, they may assort at random, thus producing as many combinations as their number permits. Both such suggestions have in fact been made; Sutton (1902), who first clearly indicated the relation between alternative inheritance and the behavior of homologous chromosome elements in maturation, rejected the first interpretation on theoretical grounds, since, according to it, individuals would have to show the characteristics of one grandparent only and not of both, whereas in fact they do show combinations of the two. While it is now known that there are possibilities of chromosome interaction during maturation which would make for recombinations of units within the chromosome, the facts of genetical analysis show that Sutton was correct in his view.

It is, however, not necessary to decide the question upon theoretical grounds, since there are observed facts to show that the assortment of parental chromosome contributions takes place by chance. Curiously enough, the first demonstration of this was made from Sutton's own slides of *Brachystola magna*. On restudying these Miss Carothers (1913) found one tetrad in which the homologues are constantly of unequal size. Since in the first spermatocyte, where these are separated from each other, the accessory, or X-chromosome, goes undivided into one second spermatocyte, it was possible to observe whether, in one individual, always the large or the small component of the tetrad accompanied it. She found in fact that it was purely a matter of chance in this case, there being equal assortments of the large and of the small element with the accessory chromosome (Fig. 23). Wenrich (1914) found similar conditions in *Phrynotettix*, and Robertson (1915) in *Tettigidea* and *Acridium*.

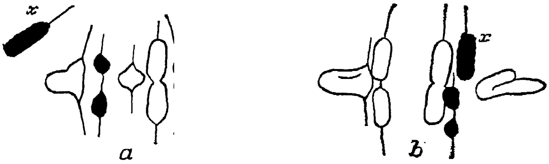


FIG. 23.—Lateral views of two first spermatocyte metaphases in *Brachystola*. The unequal tetrad and the accessory chromosome *x* in black—a few of the other tetrads in outline. In *a* the small dyad accompanies the accessory chromosome; in *b*, the large dyad (Carothers).

The conclusions established from the study of these cases of single chromosome pairs were much strengthened by a later intensive investigation of a series of chromosomes in the spermatocytes of *Trimerotropis* and *Circotettix* by Miss Carothers (1917, 1921). In the group, of which these Orthoptera are representative, there exists unusual variation in the shape of the chromosomes which, taken in connection with size differences, makes possible distinctions between members of the complex. As a result of these studies it is demonstrated that, considering the contributions of the two parents, the maturation mitoses distribute the maternal and paternal chromosomes purely by chance, so that all possible theoretical combinations are realized (Fig. 24). By an extensive statistical analysis of similar conditions in other species of this group King (1923) has confirmed the results of Carothers. It is to be noted here also that in the case of recognizable elements like supernumerary chromosomes, they also assort by chance in relation to the accessory chromosome (Carroll, 1920).

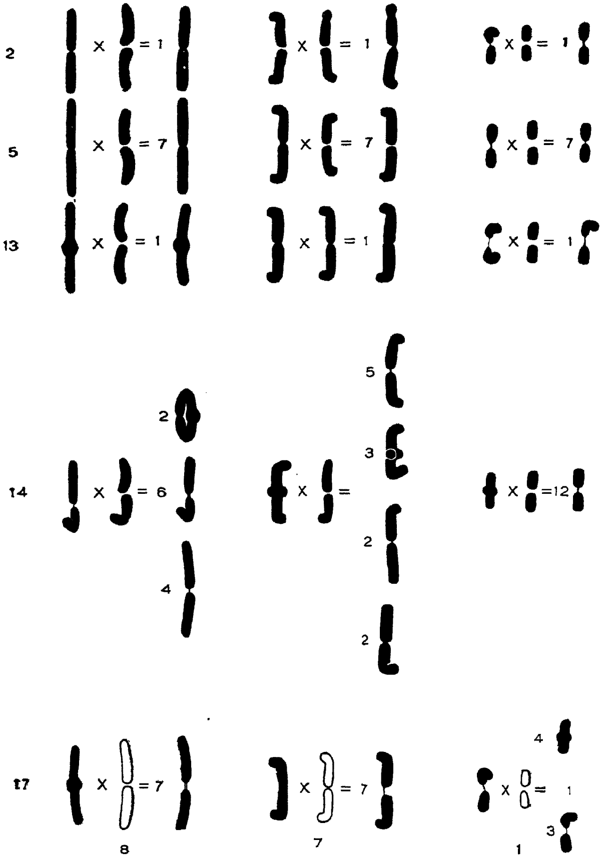


FIG. 24.—A graphic representation of the history of three pairs of chromosomes in a series of breeding experiments of *Circotettix verruculatus* by Carothers. The figures in the left column indicate the mating numbers. The three vertical group columns represent the histories of three different chromosome pairs. In each case the form of the paternal chromosome is shown at the left, the maternal at the right, and the nature of the resulting combination in the offspring after the equality sign. The number of the chromosome in the series appears at the bottom. Thus in mating 5, chromosome 1 was a rod in the father, a similar rod in the mother, and there were seven male offspring with a like chromosome form. The maternal elements in mating 17, shown in outline, were unknown.

X. THE RESULT OF THIS CHANCE AND INDEPENDENT SEGREGATION OF THE MEMBERS OF THE DUPLEX CHROMOSOME COMPLEX IS TO PRODUCE ALL POSSIBLE COMBINATIONS OF ELEMENTS IN THE DERIVED CELLS

Evidence is now overwhelming that the chromosomes perpetuate themselves in a continuous series from cell to cell and from generation to generation of organisms. All facts of order and precision in the behavior of these elements speak for this genetic continuity. From this it follows necessarily that the character of any duplex chromosome series is determined by the contributions of the gametes which originally came together to produce it. This a priori conclusion first received an adequate demonstration from the studies of Carothers (1921) already referred to. In *Circotettix verruculatus* there are three pairs of chromosomes which, from individual to individual, vary in shape owing to differences in the point of fiber attachment. By controlled matings, in which the chromosome constitution of the parents was later determined, it was possible to demonstrate that the offspring have in their cells only such shapes of these chromosomes as are contained in the parents, and the variations in their combinations are such as would be determined by chance segregation and recombination (Fig. 24).

Since this is the first clear demonstration of the chance segregation and recombination of chromosomes in a genetic series it merits detailed consideration. *Circotettix verruculatus*, an oedipodine grasshopper, appears to have only twenty-one chromosomes in its male diploid complexes, whereas the family number is twenty-three. Careful study shows, however, that there is an octad multiple involving chromosome three and another of the haploid series, which accounts for the apparent reduction (Fig. 25). Unlike most of the short-horned grasshoppers which have simple, rod-shaped chromosomes with terminal fiber attachment, *Circotettix* has a series whose shapes vary. Taken with size, this feature makes possible certain identification of three pairs of chromosomes and probable determination of the others. The shape of the chromosome is strictly correlated with the point at which the fiber running to the centrosome attaches. As thus determined there are, in *Circotettix* males, a single V-shaped accessory chromosome, three pairs of rod-shaped chromosomes, four pairs of V- or J-shaped ones, and three pairs, the members of which may vary from individual to individual, being sometimes with terminal fibers, in others with subterminal or median. The conditions of the female complex are similar except that there are two accessory chromosomes, thus raising the number of free elements to twenty-two. Numerous matings were made, and the results obtained for the three recognizable pairs were consistent throughout. For present purposes it will be convenient first to note the findings for pair number 1, the smallest and most readily identifiable element.

In one mating two of the members of this pair in the male parent were unlike, one being a rod, the other a V. This fact is most certain at the period in the germ cells when the homologous elements are joined together to make a tetrad. Just

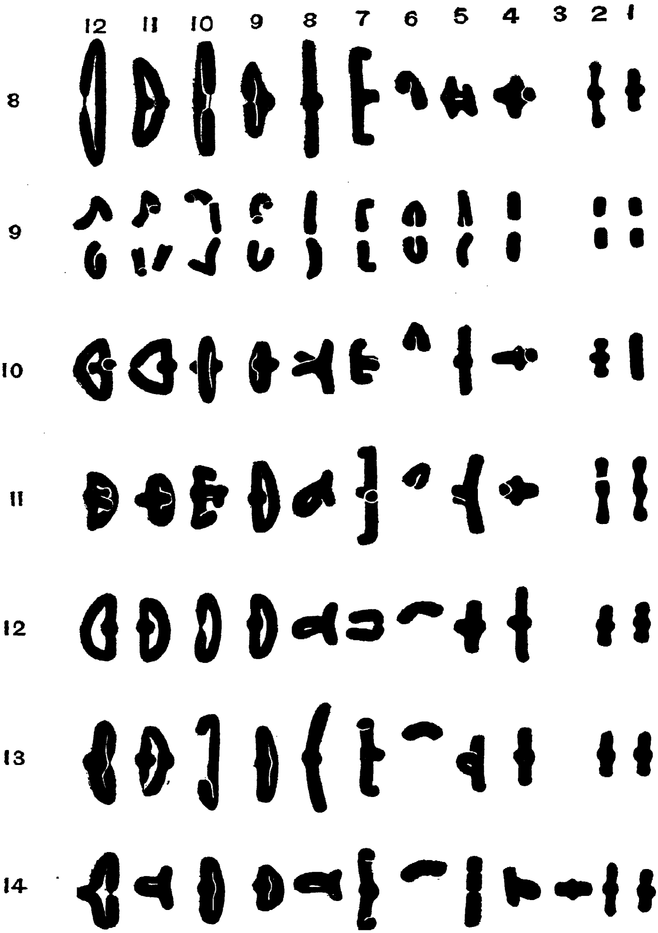


FIG. 25.—This figure shows in more detail the conditions of mating number 5 in the series of breedings of *Circotettix verruculatus* by Carothers, represented in Figure 24. The figures at the left, 10 to 14, indicate different filial first spermatocyte complexes; the ones above, 12–1, columns of homologous chromosome according to size. In row 8 is the paternal first spermatocyte chromosome; in row 9 the chromosome of a maternal somatic cell. In row 13 chromosome 11 gives indication of its multiple character, while in row 14, showing a complex of the same individual, it is manifest—chromosome number 3 being now entirely separate from the other tetrad (Carothers).

before their separation in the first spermatocyte, they are drawn out in a line and the difference in shape most apparent. The female of this mating had in her cells a pair of simple rods. Only one product of this mating was studied, and it showed in the first spermatocyte metaphase a tetrad, both elements of which were rods, indicating that in fertilization one such member had been derived from each parent to the exclusion of the possible V-shaped member in the male parent. In another mating both members of the male pair were V-shaped and in the female rods. The only possible combination here in the offspring would be that of a rod and a V, and this was realized. Still another mating had only rods in both parents, and all the offspring were of this nature.

Pair number 7 in one of the matings (14) is most convincing regarding chance distribution of homologous elements. In this case the male parent had a combination, one member of which was V-shaped and the other J-shaped, while the female had a rod and a J. There are here four possible recombinations in the offspring: a rod and a V; a rod and a J; a J and a V; and two J's. Since the four components are present in equal numbers with four combinations there should be equal representation of each association. All these expectations were realized, although the number of offspring was too small to give exact numerical ratios (Fig. 24).

This is the evidence from the behavior of individually recognizable chromosomes, but it must be remembered that they are parts of a larger group, all the members of which behave with equal constancy, one must believe, because the end result is constant. In this problem there are two phases—chance segregation of homologues in maturation and chance recombination in fertilization. These are, however, two aspects of the same process, since they are involved in maintaining a constant series of chromosomes while at the same time providing for free assortment. Thus anything showing chance assortment during maturation speaks at the same time for chance recombination later in fertilization. There is a great deal of evidence showing the general prevalence of chance in the segregation of homologues in maturation, but only a little to indicate its operation in fertilization. Taken all together, however, the evidence furnished by cytology regarding the behavior of parental chromosome complexes in maturation and fertilization strongly parallels the assortment and combination of character groups in genetical analysis.

When it is remembered that the detailed genetical work upon *Drosophila* shows that the same structural feature of the body is influenced by factors in different chromosomes, it is apparent that variations in the combinations of these must definitely affect the course of development in any part of the body. Disregarding the possibility of internal reorganization of the chromosomes during synapsis, it is apparent that with any considerable number of chromosomes in the complex chance assortment and recombination affords a means for extensive variation in reproduction. This aspect of the subject was considered by Sutton (1902) when he first suggested the parallelism between Mendelian phenomena and chromosome behavior. The possibilities of combinations are shown in his table:

CHROMOSOMES		COMBINATIONS IN GAMETES	COMBINATIONS IN ZYGOTES
Somatic Series	Reduced Series		
2	1	2	4
4	2	4	16
6	3	8	64
8	4	16	256
10	5	32	1,024
12	6	64	4,096
14	7	128	16,384
16	8	256	65,536
18	9	512	262,144
20	10	1,024	1,048,576
22	11	2,048	4,194,304
24	12	4,096	16,777,216
26	13	8,192	67,108,864
28	14	16,384	268,435,456
30	15	32,768	1,073,741,824
32	16	65,536	4,294,967,296
34	17	131,072	17,179,869,184
36	18	262,144	68,719,476,736

While we are as yet largely ignorant of the degree and character of somatic influence upon the hereditary materials of the germ cells, it is clear enough that these expressions of specific organization are not independent of each other. The individual, in the beginning a single cell, has its material organization very early differentiated into cell groups which build up the body and carry out its processes, and into germ cells, which apparently serve no direct purpose in individual existence, but which look forward to succeeding generations. Still, these two great classes of cells make up the body of the organism, and the germ cells are dependent for their existence upon the functions of the body cells; and the media with which they work in reproducing themselves, often into numbers reaching to the millions in a single animal, is supplied by the body cells. It is inconceivable that in an adaptation so fine and precise as this there should be no mutual influence between the members. On the other hand, the germ cells are old and strongly established in their characters—the resultants of millions of years of repeated experiences which are registered in their structure. It is not to be expected that a single one of this almost infinite series of incarnations can have any profound effect upon normal processes. Diseased conditions of the body may register in the germ cells for a time, but the flow of normal processes continues through the unending centuries, reproducing again and again their type of organization. *Lingula*, a brachiopod, after reproductions in numbers so great as to be entirely beyond the range of human comprehension, presents the same characters as it did in the Cambrian period. Meanwhile, any number of other species have arisen and gone out of existence or become modified in form. It must be true of the relation between body and germ cells, as it is of all other organic relations, that they differ from individual to individual, and from group to group. That the germ cells should exist in any way apart from and uninfluenced by the conditions of the complex of which they are a part is inconceivable.

If the germ cells are, then, a register of racial experience, it is clear that a mechanism for assorting and recombining the pages of this story would tend to an ultimate uniformity of expression with almost all degrees of individual variation. Such possibilities seem to lie within the nature of the chromosome behavior in maturation and fertilization. The haploid group of either parent cell contains the full register of class characteristics, and, upon fertilization, the individual is provided with a double record. In its germ cells the double pages are separated and assorted by chance into different units, so that, if there are but eighteen of these pages in the single series, the possible varieties of it are 262,144. When later fertilization combines these in duplicate, the possible expressions of variation reach the incomprehensible number of 68,719,476,736. It is a matter of common observation that no two individuals of a species are ever exactly similar in the embodiment of its characteristics; just as definite is the evidence that the same story of organization is revealed in each instance. Always it is a question of balance, and of emphasis upon one feature or another of the same story. Thus the evidence from the operation of hereditary processes and from the behavior of their mechanism is at one in the demonstration of the possibilities of almost infinite variety in the expression of a common series of characters. If even the slightest effect of bodily experiences can be impressed upon the appropriate germ-cell determinant, the means for its possible influence and perpetuation are provided in the permutations of the chromosomes in maturation and fertilization. Here it must be remembered, however, that the experiences of the germ cells of any one individual are widely different in relation to the body, since age and all the circumstances of life are variable.

XI. IN PREPARATION FOR THE PROCESS OF SEGREGATION IN MATURATION, THE MEMBERS OF EACH CHROMOSOME PAIR COME INTO INTIMATE PHYSICAL CONTACT BY SYNAPSIS AND HERE MAY UNDERGO MUTUAL INTERACTIONS OF SUCH A NATURE THAT THEY ARE VARIOUSLY ALTERED IN CHARACTER

The process of sorting which operates in the maturation divisions serves only to alter the character of combinations—it does not affect the nature of the units entering into them. Preceding such assortment of chromosomes they pass through a series of changes highly interesting in themselves and most suggestive of functional significance. A study of this period in the history of the germ cells is fraught with the greatest difficulty, both on account of the complexity of the changes involved and the minute size of the structures taking part in them. It is safe to say that no subject in cytology has been so bedeviled by faulty and imperfect work as this. In the literature may be found expressed views which reach approximately the limit of theoretical possibilities. The existence of this diversity of opinion is indicative, not only of varied abilities among the writers, but also of the difficulties inherent in the subject. Of all the multitudinous papers extant, therefore, only a few bear the stamp of authority impressed by the evidences of care, experience, understanding, and judgment necessary for such studies. The account which follows will attempt to approximate the general outline of a picture which would reflect the judgment of the majority of competent authorities.

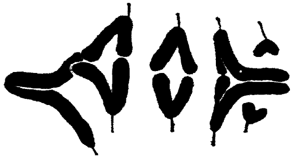


FIG. 26.—Early anaphase of first spermatocyte of *Mecostethus* showing the four chromatids in the tetrads. Fiber attachment is terminal in the diploid chromosome, and the apex of each V represents the point of contact between two such elements (McClung).

It is admitted by practically all familiar with the phenomena of maturation in the germ cells that the end result is to reduce the chromosome number to one-half the normal; and it is also generally agreed that preceding the actual material diminution of the chromatin elements, there is a so-called pseudo-reduction, during which the separate chromatin bodies are present in the reduced number, but so combined as to include all the original series. Clearly then the problem to be solved is the nature of these unusual chromosomes and the manner of their separation, always assuming that the individuality of each element is maintained and perpetuated.

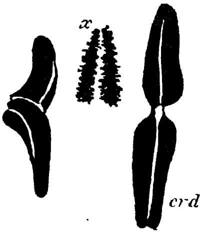


FIG. 27.—The accessory chromosome and two tetrads of *Amphitornus* at the beginning of the anaphase. The chromatids of the accessory chromosome, *x*, are more widely separated, and more clearly show the composition of the V elements of the tetrads *crd* (McClung).

There are many aspects to the synaptic process, but in the present discussion emphasis will be laid upon those which relate most intimately to genetics. As a matter of convenience of presentation also, the stages will be considered partly in reverse order, commencing with the elements as they lie in the anaphase of the first meiotic division, it being understood that essentially the same conditions obtain in both spermatogenesis and oögenesis. Since also all the chromosomes in the complex, with certain exceptions to be mentioned, behave practically alike, the history of a single one of them will be followed. The orthopteran chromosome in spermatogenesis will serve as the type. Regarding such an element as it lies thus divided, we see that the two halves are V-shaped structures and that the limbs of each V are separate, equal elements, i.e., each has two parts and the two V's together, four of practically equivalent dimensions (Figs. 26, 27). The following, second spermatocyte division (Fig. 28), with which we are not now concerned, completes the separation of the limbs of the V's, and places them in different cells. The four spermatids thus produced contain, each, one limb of one V (Fig. 29).

Since it is merely by a slight movement in the early anaphase that four parts are revealed in each chromosome, it must be assumed that the compact metaphase chromosome just preceding contains these separate divisions although they are not fully manifest. The diversity of external form in the metaphase is very considerable in the first spermatocyte chromosome, even in a single cell, and the same element may vary in different cells, but there is an underlying similarity of shape in them all. It is clear, therefore, that variation of form does not mean difference of structure, and an intimate acquaintance reveals the fact that it expresses merely changes in the space relations of the four constituent parts, or chromatids. The simplest disposition of these occurs in *Mecostethus*, where all the metaphase chromosomes are of about the same shape (Fig. 30). Here the four chromatids lie extended in the equatorial plate to form a rod with a slight enlargement in the middle where the fiber attaches on each side. This point corresponds to the apex of the V in the anaphase following. If the halves of the rod swing around into a circle, a ring is formed, and this is a common form in many animals (Fig. 31). If the angles where the fibers attach move toward the poles of the spindle, a cross is produced which may have equal arms, or a long one in either direction. Sometimes, in favorable cases, the external form betrays something of the internal structure—a clear, diamond-shaped area in the middle of the chromosome marks the point of union of the four chromatids, a notch at each end indicates the line of division between the two, and sometimes this merges into a lighter line extending the length of the chromosome to the diamond-shaped area at the center. These structural conditions indicate clearly that the metaphase chromosomes, or tetrads, are made up of four parts, and that these are most intimately associated to form this unit element in mitosis.

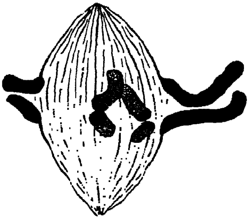


FIG. 28.—Lateral view of second spermatocyte metaphase with four dyads drawn. The two central ones have their chromatids still in contact at the apex of the V, which now lies on its side, while the lateral ones are beginning to move apart (McClung).

The questions of greatest interest theoretically regarding the tetrad are: (1) What are the sources of these four constituent chromatids? (2) What are their resemblances and differences? For answers to these questions we require the full genetic history of the elements, but there are some suggestive facts revealed in the first spermatocyte metaphase. One of the most obvious features here is commonly the exact size equivalence of the four chromatids. While the various tetrads show marked differences in size their constituent parts appear equivalent. Again, in the matter of shape there is almost complete uniformity, and finally in their behavior during division there is agreement. All these facts, applying criteria used for determining relations between entire organisms, speak for organic relationships of the most intimate character, i.e., we judge these four chromatids to be as nearly identical as four physical objects of these dimensions can be.

Occasionally, however, there are departures from this uniform system which are most helpful and suggestive. Wenrich (1916) has described in *Phrynotettix* a tetrad two of whose chromatids are much smaller than the other two, and these are moreover granular in character as opposed to the clear, homogeneous appearance of the larger two. Very curiously also the first meiotic division separates the four chromatids so that in half the cases the two large and two small ones remain together, and in the other half one large and one small one are united (Fig. 17). Carothers (1913) described in *Brachystola* a tetrad of unequal parts, but in this case like pairs remain together. Later (1917) she described in *Trimerotropis* a series of tetrads the pairs of whose

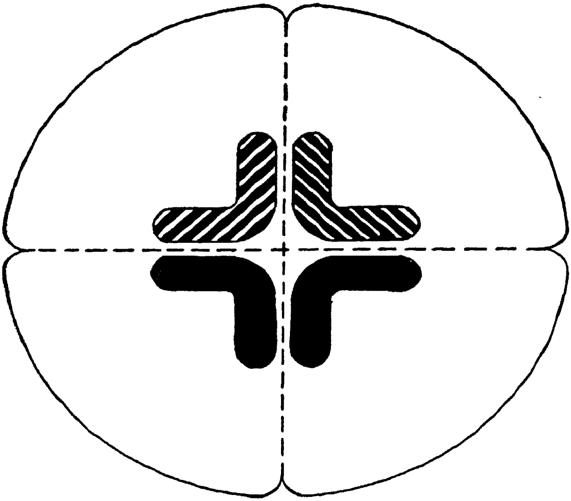


Fig. 29.—A diagrammatic representation of the fate of the four chromatids of a tetrad in the two maturation divisions. The circular outline would represent the first spermatocyte; the quadrants the four spermatids produced. Into each of these would be distributed one chromatid of the tetrad. The elements in black would be from one parent; the ones shaded from the other. The dotted lines between the quadrants indicate also the planes of separation carried through the tetrad by the maturation divisions. It is a matter of chance which comes first.

chromatids differ in shape—a pair of V's being united to a pair of rods or J's—and these occasionally being further differentiated by constantly appearing constrictions in one pair (Fig. 32).

Since the early investigations upon maturation, it has been the assumption that one pair of chromatids represents the paternal contribution to the tetrad and the other pair the maternal. This unusual chromosome would then be the sum total of the two parents' contribution of a given grouping of chromatin materials brought into the closest possible relations and smallest compass. Similarities of form, size, structure, and behavior would speak for uniformity of nature in the four parts; diversities in these matters, on the contrary, would be indicative of differences in the parental contributions. All the facts reviewed under other divisions of this section regarding the differentiations and behavior of chromosomes support this idea. Wenrich's observation upon the unequal tetrad was very suggestive of differences in the parental contributions. But it was not until Miss Carothers (1921) traced recognizable groups of chromosomes from parents to offspring that it was demonstrated that two of the chromatids in each tetrad are definitely paternal and the other two maternal. It may now be confidently asserted that what was long suspected from observations upon the equivalence in the chromosome contribution of the two parents in fertilization and the likeness in the process of maturation in the two sexes

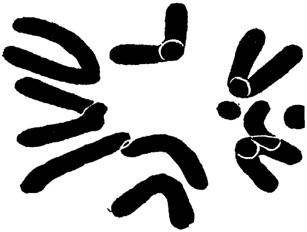


FIG. 30

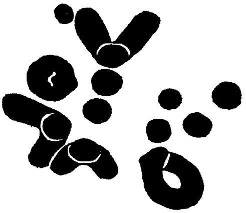


FIG. 31

FIGS. 30 and 31: FIG. 30.—Polar view of first spermatocyte metaphase of *Mecostethus*. The slender, rod-shaped tetrads of this genus lie fully extended in the equatorial plate (McClung). FIG. 31.—Polar view of first spermatocyte of *Mestobregma* with tetrads in the form of rings, V's, and rods (McClung).

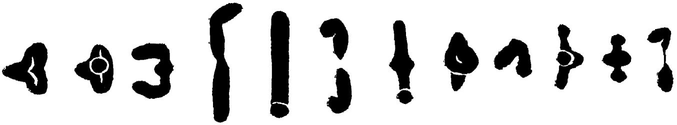


FIG. 32.—A first spermatocyte complex of *Trimerotropis fallax*. The homologous dyads lie above and below the median constrictions, and it is noticeable that they are frequently of dissimilar shapes (Carothers).

has been definitely proved by the work of Miss Carothers. We now know that the tetrad represents a union of two chromatids from the father with two similar, but sometimes recognizably different, ones from the mother. All the behavior of the tetrad, then, must be interpreted upon this understanding of its structure. Because the two meiotic divisions separate these four chromatids and place them in different cells it is plain that two of these receive paternal elements and the other two maternal. When, however, the result of this process is tested out by genetical experiments, it is found that no two of the multitudinous germ cells produced by any two individuals ever give the same combinations of character, although if more than one organism is produced from a single zygote they approach identity very closely. It must be, therefore, that somewhere in the preparation of the germ cells for union they undergo a reorganization which differs in each particular case. Extensive genetical investigations by Morgan and his co-workers have revealed much of the nature of this reorganization, as will appear later. It is sufficient here to note that these results indicate a physical interchange between homologous chromosomes at equivalent regions in their length. Such is their nature as to suggest the need for the closest physical association. Using temperature to affect the egg recognizably, Plough (1917) has found by noting the rate of its development that the time of this interaction comes at a period much earlier than the first maturation metaphase. It will be of interest, therefore, to trace back the history of the chromosome to find a period at which their structure would offer the best physical conditions for mutual interactions.

In the metaphase, as we have seen, the four chromatids are strongly concentrated and homogeneous in structure, i.e., their constituent elements, or chromomeres, are much compressed, their surface reduced to a minimum and movement restricted or inhibited. The changes leading up to this state of concentration and immobility represent progressive approaches to it from an almost opposite one of extension and mobility. Thus, just before the metaphase, the tetrad is found approximately of the same shape and proportions, but with its substance diffused so as to show large granules. At this time the four chromatids are clearly distinguishable, lying in the same relations as their outlines in the metaphase suggest, and their constituent chromomeres may clearly be seen and compared in size and position in each of the four elements. Wenrich (1916) has shown that at this time the smaller size of one pair of chromatids the unequal tetrad of *Phrynotettix* is due to the absence of one large chromomere in each. A little earlier there is an extreme state of diffusion, so that the chromosomes appear almost to fill the nucleus and to be in intimate contact with the karyoplasm. At this time the chromosomes are scattered through the nucleus without regular arrangement, but intimately attached to the nuclear membrane over much of its surface. This is during the period of rapid cell growth, and all the conditions would suggest a form of chromosome best adapted to take part in metabolism.

Preceding this stage of chromatin diffusion and irregularity of position, comes one which shows each chromosome as a loop with the two ends attached to the nuclear membrane near one side of the nucleus (Fig. 33). The chromosome thus being drawn out to a considerable length shows its chromomeres widely separated and arranged in a double row. The exact correspondence of the chromomeres in size and position is such as to suggest very strongly the longitudinal splitting of the chromatin thread in an ordinary mitosis, and this was at first the only interpretation offered. It would seem now, principally through the studies of Wenrich (1916) on *Phrynotettix*, where there are recognizable distinctions between the homologous chromosomes, that this double thread is really the pair of chromosomes lying side by side with the equivalent chromomeres opposite each other. There are many investigations pointing to this probability, but the only case in which individual homologues have been recognized is that of *Phrynotettix*. In the spermatogenesis of the amphibian, *Batrachoseps*, the stages are serially arranged along the testis, and it there appears that, preceding this double loop, there is a gradual approximation of two thin threads, beginning at both ends of the loop which lie together at one pole of the nucleus. These conditions have been worked out in detail by Janssens (1905). Similarly, the Schreiners (1905) have shown that in the annelid, *Tomopteris*, a thick double loop is produced by the coming together of two thin threads of equivalent dimensions. More recently Gelei (1921) has traced in the oöcytes of the flat worm, *Dendrocoelum*, the course of each of the fourteen loops in the thick thread stage (Fig. 34). There are many more or less convincing cases which seem to indicate the approach side by side of thin threads to form the thick loop. In all of these cases the difficulty has been to determine between the alternatives of approximation of homologous chromosomes and the reunion of the split halves of a chromosome.

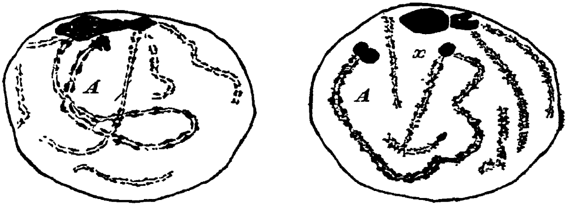


FIG. 33.—Wenrich's chromosome "A" in *Phrynotettix*, during the late prophase in the form of a loop. The constituent chromomeres are apparent. The accessory chromosome, *x*.

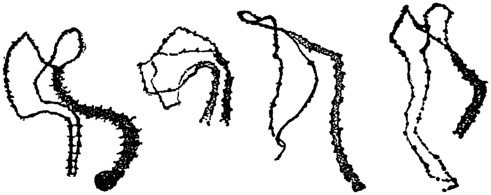


FIG. 34.—First spermatocyte chromosome loops of the flat worm, *Dendrocoelum*, with the thin threads in contact throughout part of their length (Gelei).

Wenrich found in *Phrynotettix* chromosome pairs, the members of which are distinguishable apart, and it there appears with great clearness that these unequal homologues lie side by side, and that they are comparable granule by granule except that the terminal one is lacking in one member.

The chromosomes of *Phrynotettix* and other Orthoptera at this stage reveal another structural condition that is of the greatest importance. It has long been known that in the first spermatocyte of these animals there are chromosomes in the form of multiple rings so constituted that each successive ring lies at right angles to the preceding. In the stage just before the metaphase, where the constituent chromatids are visible, it is clear that the rim of each ring is double and that the cleft in the wall of one annulus becomes the included space in the next. Such an effect would be produced by taking four flexible rods of equal length, lying side by side, and separating two of them from the other two for a short distance to form a ring and then in turn separating the other two in a plane at right angles to that in the first ring. As many as three or four rings thus disposed are found in the first spermatocyte chromosomes of various Orthoptera, *Batrachoseps* and other forms. Janssens considers that at the points where one ring merges into another there is a crossing-over of one thread to combine differently in the next loop and that subsequently a new plane of division cuts through these chiasmas and makes new combinations. Nothing is clearer in chromosome structure in the Orthoptera than that the original planes of separation between the four chromatids are maintained, and that they constitute those along which cleavage occurs in the two meiotic divisions. It is true that in the greatly extended conditions the thin threads roll on each other, but there is absolutely no evidence that they break and recombine as Janssen's chiasmatype theory demands.

An explanation for the formation of these multiple rings is supplied by the structural condition of the chromosomes in the thick thread stage of the first spermatocyte of *Phrynotettix*. Here it is observed that the chromosome has first a cleft beginning at one end and another at right angles to this at the other end. A continuation of these divisions produces four rods which, in the short chromosome where the process is clearest, diverge strongly along one of these planes of cleavage producing a V-shaped chromosome, the arms of which are divided along the plane of the cleavage beginning at the opposite end. In longer chromosomes, separation occurs at different levels, as described in the hypothetical case of the rods above, and in the planes at right angles to each other, producing the multiple rings. These are seen with great clearness in many tryxaline grasshoppers.

What are these planes of cleavage lying at right angles to each other in the first spermatocyte chromosome? So far as their later history is concerned, it is clear that they are the ones along which separation of the four chromatids takes place in the meiotic divisions following. Regarding their origin there is less certainty. Are they *de novo* divisions cutting through the chromosomes or are they expressions of inherent structural conditions? All the evidence at hand indicates that they are the latter and that one plane represents such a cleavage as chromosomes commonly show in their reproduction and the other a reappearance of the space between homologous chromosomes temporarily obliterated by their close union. The evidence for this distinction is clearest in the differential chromosomes of *Phrynotettix* where the probable parental elements differ in size and behavior. In this case it is seen that one of the planes falls between the unequal pairs of chromatids while the other separates those of equal size. Assuming that the large pair comes from one parent and the small pair from the other, as the work of Miss Carothers justifies, then it is plain that the cleavage between these separates parental chromosomes and is a reduction, or segregation division, in technical language. The other division is such as commonly occurs in chromosomes at mitosis and is, therefore, termed equational.

The stages leading up to the one just considered have not been heretofore described in a satisfactory manner. They have been the object of study by the author for many years, and only recently has material exhibiting them with any clearness been available. It will require much more study on less satisfactory material to determine the variations of the process as manifested in the tryxaline, *Mecostethus*. A brief outline of these early changes in the chromosomes will, however, be helpful in gaining an appreciation of the nature and complexity of the union between homologous parental contributions in the stage of synapsis (sometimes called syndesis) and in this consideration it will perhaps be best to follow the sequence of changes leading up to the four-rod condition instead of continuing the reverse order.

All the changes so far considered have fallen in the generation of cells called the first spermatocyte, and it is true that the ones involved in each synapsis occur in such a stage, but they run back into the telophase of the last spermatogonial division indistinguishably. The question at issue here is: Do the chromosomes at this period already exhibit a longitudinal division? This is by no means an easy matter to decide, for, at this time, the chromatin is coming out of the condensed state of the metaphase, taking up fluid, sending out processes and becoming a part of the vesicular chromosomes so characteristic of the orthopteran elements of this phase. Nevertheless, after long-continued study and after meeting certain unmistakable cases, it seems to the author highly probable that the chromosomes enter into the changes of the first spermatocyte prophase already longitudinally split as they would be in preparation for an ordinary mitosis. At this time the nucleus constitutes almost the entire cell, and all the chromosomes run approximately parallel directly across it. Very soon, however, the accessory chromosome, being unpaired and therefore not involved in synapsis, begins precociously a series of maneuvers which, in general, foreshadows the movements of the euchromosomes. Its successive changes, therefore, serve as a guide in the seriation of the chromosome movements during synapsis. In brief, the accessory chromosome, lying in its own separate vesicle, first elongates, becoming contorted, and then has one end turned back to the opposite side of the nucleus thus forming a complete loop with the two ends in contact (Fig. 35). The vesicle sometimes breaks, and then the accessory chromosome may completely encircle the nucleus, but ordinarily it lies at one side as a pear-shaped structure with somewhat indistinct indications of its looped structure. Meanwhile, the cell has been growing and the other chromosomes still stretch across the nucleus, but with some contortions due to their increasing length.

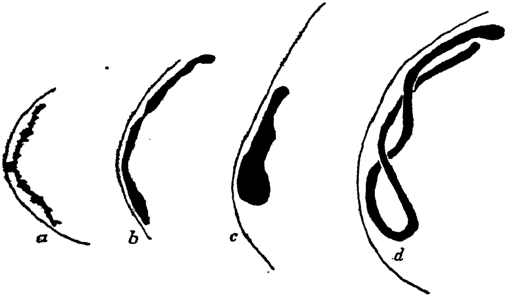


Fig. 35.—Successive stages in the movement of the accessory chromosome during the first spermatocyte prophase of *Mecostethus*. Just after the last spermatogonial division it reaches directly across the nucleus, *a*. With the growth of the cell it elongates, but almost immediately one end turns sharply and glides back upon the shaft of the chromosome, *b*. This results in an apparent shortening of the element, which is now pyriform in shape, *c*. Later it again elongates and sometimes shows the separate limbs of the loop, *d* (McClung).

In *Mecostethus* there now occurs a rather sudden reduction in the number of separate chromatin bodies from twenty-two (leaving the accessory chromosomes out of account) to eleven, and in favorable preparations it is seen that these are made up of two irregular rods which twine about each other irregularly (Fig. 36). That is to say, thus early in the first spermatocyte, while the chromosomes still retain the position of rods extending across the nucleus, as they did during all the spermatogonial divisions, they pair off and reduce the number of separate chromatin masses to one-half. Certain of the smaller chromosomes at this time move precociously and may thus be recognized. In these cases it is apparent that the two rods in the group are of equivalent length, and have the same number of chromomeres with the same spacing (Fig. 37). Certainly in these cases, and probably in all, homologous chromosomes have come together to effect the reduced number. These double chromosomes now follow the action of the accessory chromosomes, and form loops apparently in a very

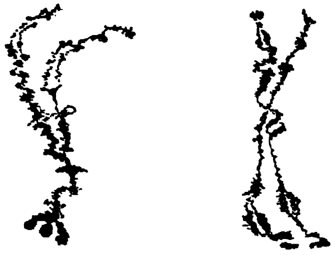


Fig. 36.—Early stage in the approximation of the homologous chromosome of *Mecostethus* to form the tetrads of the first spermatocyte. Indications of the longitudinal split in each member of the pair are seen in various places (McClung).

similar way (Fig. 38). All the chromosomes remain attached on one side of the nucleus to its membrane, while the other end of each rather suddenly sweeps around to this side (Fig. 39). The result is to leave behind a thin thread reaching approximately across the nucleus and connecting with the heavy mass which completes the loop. Gradually the chromatin equalized throughout the length of the chromosomes, which is greatly increased, so that soon the nucleus seems filled with a tangle of thin threads or even with immense numbers of apparently unrelated fine granules. While the cell increases rapidly in size these greatly extended and convoluted loops become shorter and heavier, until the stage of the thick, double thread is reached, and this is soon followed by the stage in which indications of a split at right angles to this more pronounced cleft appears—as has already been described.

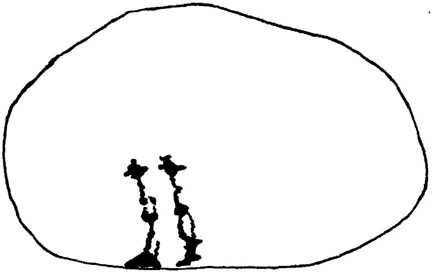


FIG. 37

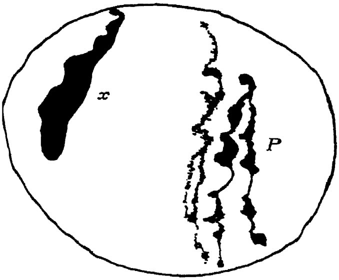


FIG. 38

FIGS. 37 and 38: FIG. 37.—A small precocious pair of chromosome in *Mecostethus* lying side by side and showing corresponding chromomeres (McClung). FIG. 38.—A first spermatocyte nucleus of *Mecostethus* in which the accessory chromosome, *x*, has completed its loop, while one of the tetrads, *p*, somewhat precocious, is just being formed—its members still extending directly across the nucleus (McClung).

Now it must be apparent that if the chromosomes were already longitudinally split and then became associated side by side, the very thin thread left behind by the sudden withdrawal of the paired chromosomes to form the loop must have within it these planes of separation as would also the very fine convoluted loop later filling the nucleus. But, since this thin thread itself approaches the limits of microscopical vision, no trace of these clefts is, of course, visible. Later on, there reappears in the chromosomes two longitudinal divisions at right angles to each other, and, unless it be assumed that these are *de novo* formations, we must conclude that they are the persistent planes of the early stage. There is really little assumption needed here in the case of the differential chromosomes of *Phrynotettix* where one plane divides the large and small members into equal parts and the other falls between them. It is true, however, that at one time visible continuity of cleavage planes in the chromatin thread is lacking, and those who wish to consider this as evidence of their loss will do so, but they must disregard the parallel conditions which exist before and after this period.

One thing is plain, however—within this exceedingly slender thread, the substance of a pair of chromosomes is drawn out to great attenuation and into most intimate contact. The homologous contributions of the two parents here achieve the maximum degree of association in a linear series. It has long been assumed that the factors or genes governing development of characters have a linear arrangement; the work of Morgan and his associates demonstrates this, and shows that the number of characters in a group corresponds with the linear dimensions of the chromosomes with which they are associated. In general, the groups of characters entering into an individual emerge in similar association in its offspring, but in the female of *Drosophila* they undergo a series of mutual exchanges between the homologous groups of the two parents so that the offspring show various assortments of common, but contrasting, associations. If the grouping remains constant, characters are said to be linked; if mutual exchanges occur, the phenomenon is called crossing-over.

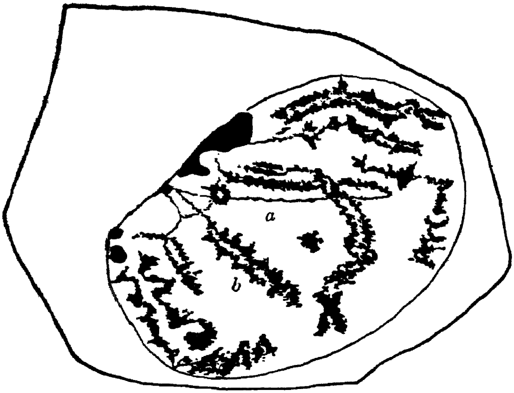


FIG. 39.—Later stages in the movement of the tetrads of *Mecostethus*. At *a* one of these has completed its loop, but one half, which has been left behind by the sudden movement, is very thin. The chromosome marked *b* has just started this movement while others have not even begun it (McClung.)

The number of separate groups of characters linked together in *Drosophila melanogaster* is four—the number of chromosome pairs is four, the groups of characters are of unequal numbers—the lengths of the chromosomes are of corresponding proportions. The character groups of parents assort by chance in their offspring—their chromosomes thus assort in maturation of their germ cells. The assumed linear arrangement of genes with differences in distance corresponds to observed structural relations of the chromomeres in chromosomes such as those of the Orthoptera. Mutual exchanges between linearly arranged and discrete masses of material conceivably could best take place at their greatest extension and approximation; the experimental work of Plough on *Drosophila melanogaster* indicates that crossing-over occurs at a period in the germ cells corresponding to that at which the greatest linear extension of the chromosomes comes about.

The conditions of biparental inheritance are very complex and their significance by no means fully appreciated. Since such a form of reproduction is practically universal in the higher animals it is certain that, in some way, it is bound up with a high degree of complexity and differentiation. In what way participation of two parents in the act of racial perpetuation should be involved in continuing and increasing complexity is not yet clear, but it is suggestive that the equivalent chromosome contributions of the two parents, by random assortment in maturation and chance recombination in a series of offspring, offer the greatest opportunity for testing out combinations of characters against the conditions of existence. If innumerable repetitions of a given series of processes tended to increase their precision unequally among different groups of cells thus leading to differentiation of functions, the repeated sortings and recombinations of the chromosomes in biparental reproduction would afford opportunity for recording these experiences in the largest possible number of combinations.

If now we add to the mechanism of assortment and recombination afforded by the maneuvers of the chromosomes the possibility of an inner reorganization of each chromosome by most intimate physical association with another of identical structure but slightly different history, there is provided at once a means for testing out each new set of combinations and of rejecting the incompatibles. That synapsis is such a test of incompatibility is shown by its failure to function normally in hybrids of wide crosses.

A comparison of the results of vegetative propagation in plants with that of seed reproduction is very suggestive of the changes which have been noted in the chromosomes of the germ cells. By the former method a continuous succession of individuals can be produced, each of which presents an almost identical combination of characters. If on the contrary such a plant produces seed and these develop, the resulting progeny are of great variety. In terms of the chromosome theory these conditions would seem to indicate that, so long as the diploid group is maintained unaltered, the results of its operation show little variation, but when synapsis, segregation, and recombination occurs in the germ cells the end results are always different in some degree. So far as these effects are concerned it would seem that chance segregation and recombination could do little more than affect the groupings of characters, although this in itself might be productive of great variation in the offspring. Synapsis, on the other hand, opens up the possibility of real changes in the nature of the chromosomes. If at one level of a chromosome there may be three or more possibilities introduced from different parents and some of these may be determiners between life and death, it is evident that there is great significance in the changes which the chromosomes may undergo.

XII. THE CHROMOSOMES OF THE EGG AND SPERM, HAVING THUS BEEN MODIFIED OR TESTED IN SYNAPSIS, AND INDEPENDENTLY ASSORTED BY THE MATURATION DIVISIONS, HAVE THE DUPLEX CONDITION RESTORED IN A NEW INDIVIDUAL BY FERTILIZATION

In a preceding division (VIII, p. 637), the evidence for the correspondence in character of the chromosome contributions of the parents to the new individual has been given. By variations in size, form, and behavior, the particular source of each element may sometimes be recognized. Equivalence in the number and size of maternal and paternal chromosomes is very apparent here, and were the elements invariable in their character, under a fixed environment, the product of their action should always be the same. As a matter of fact, however, there is never equivalence between individuals and we must assume that, in their inner organization, the chromosomes always differ in some degree. Every fertilization, therefore, is a new experiment in combinations of characters to be tested against the environment. In the preceding maturations of the ovum and sperm the old combinations, after the intimacies of synapsis, are broken up and they then bring their time-tried products together for a new effort by fertilization. Although this same process, with apparently identical materials, has been repeated and repeated until the numbers are totally beyond comprehension, never before have exactly the same records of experience been thus combined. Here is a single cell whose rate and character of reproduction we may exactly anticipate, if we know its kind, and yet, when all its manifold progeny are molded into the shape of a finished body, the result in each case is something in a measure unique.

XIII. CONTRIBUTIONS TO THE NEW INDIVIDUAL BY EGG AND SPERM ARE ALIKE IN ONLY ONE RESPECT—THE CHROMOSOME SERIES—BUT THE INFLUENCE UPON BODY STRUCTURE IS ESSENTIALLY THE SAME

If there were no distinction between the functions of the different parts of the cell, if mass of material alone were the measure of influence, there would be no comparison between the action of the egg and sperm in heredity because the sperm is always relatively minute. At the time of fertilization, however, it is manifest that the two germ cells are almost exactly equivalent in the chromosomes they contribute to the new individual. If, as in *Ascaris megalocephala* var. *univalens*, there is a single chromosome in the sperm pronucleus, the egg pronucleus will have one of essentially the same character; if the sperm pronucleus have two, as in the variety *bivalens*, their mates will be found in the egg pronucleus. There are a great many known cases of fertilization in plants and animals now, and they always show, aside from the sex-determining mechanism, this equivalence between sperm and ovum regarding their chromosomes.

Corresponding to this equality in their chromosomes, the sexes show a like equality in their relation to the characters of the offspring. So far as genetical results indicate, character controls are indistinguishable in number and value in the germ cells of the two sexes. Whether a character enter through the male or female line is a matter of indifference. This is true even of those features which mark the sexes apart. In crosses, the differentials of the male sex may be borne by the ovum in the combination. As has already been noted, what is true of the individual character controls is true also of the entire group, for it has been demonstrated that either haploid group alone is sufficient to condition full development.

XIV. WHILE THE SPERM CONTRIBUTES LITTLE BUT CHROMATIN, THE EGG SUPPLIES THE CYTOPLASMIC ENVIRONMENT AND FOOD. THESE MATERIALS, HOWEVER, HAVE BEEN LAID DOWN UNDER THE INFLUENCE OF A DUPLEX SERIES OF CHROMOSOMES DERIVED FROM MALE AND FEMALE LINES

A series of excellent investigations by Wilson (1904) upon mollusks and annelids, and by Conklin (1905) upon ascidians and other forms, have shown very clearly that there are definitely localized materials in the egg which are exactly employed in the construction of various parts of the body, like the muscles, nervous system, alimentary canal, etc. This question will be considered at length in the section devoted to the subject. Because the egg thus supplies the material out of which are fashioned fundamental systems of the body, it has been suggested that it plays a preponderating rôle in heredity. Loeb (1916) suggests that there are underlying racial characters in the cytoplasm and that the chromosomes govern only individual or varietal characters. Conklin also holds that there are differences in the contributions of the sexes and that the type of early development is set entirely by the egg. The implications of these views are that characters are determined otherwise than by action of the chromosomes, and that the individual can be separated into different types of characters. While it is true that in genetical work external or surface structures have largely been used as indexes of the hereditary mechanism at work, it is not true that these are superficial or unimportant. As has been pointed out by Morgan and others, they are often only one expression of a force that influences much of body development. Some of them determine such a fundamental feature as additional appendages in *Drosophila* or even the question of survival or death. It is illogical and against the evidence of development in organisms to separate their structural features into categories of racial and specific characters.

The fundamental objection to these interpretations, so far as they concern the present discussion, is that they assume the presence in the egg of substances over which the chromosomes have little or no influence and which are, therefore, of a different character fundamentally from those directly related to the chromatin. Now the generally accepted view of the cell is that it is a structural and functional unit and that its parts work together in a co-ordinated manner in the performance of its functions. Experiments have demonstrated that the nucleus is indispensable in the functioning of the cell. Genetical experiments have fixed the locus of character control during development in the chromosomes, and have shown the parallelism between the nature and behavior of the chromosomes and the appearance of structural and functional characters. The question is no longer: "Do the chromosomes act as character determiners?" but rather "How do the chromosomes act as character determiners?"

It is perfectly obvious, of course, that the final result in any reaction depends upon the nature of the elements entering into it and the conditions under which they operate. Change any one of these factors and the result is altered. The chromosomes in some way direct or govern the rate and character of the cell's operations; the result depends upon the nature of the chromosomes, the materials with which they react, and the physical conditions of the reactions. A difference in any one of these means a changed product. In a certain strain of *Drosophila* at normal temperature, the individuals have the typical three pairs of appendages; rear them at a lower temperature and a fundamental hexapod character disappears in the presence of multiple appendages (Hoge, 1915)—an external condition has modified development. Select another strain of *Drosophila* with known chromosome factor differences, and no possible temperature changes consistent with development will affect the number of appendages—an internal element governs the nature of the product. But vary the combinations of characters as you will, distort, disturb, and damage as you may, a *Drosophila melanogaster* egg produces only a fly with the structural complex of the species, however much it may be modified. The mechanism which governs the appearance and permutations of several hundred characters in this insect has been subjected to a painstaking analysis and its operations are as definitely known as any facts in biology. The relation of the chromosome mechanism to character control has been established. A full discussion of the genetical evidence on this subject appears in another section.

How then can the existence of a controlling nuclear mechanism be reconciled to the presence in the egg of definite regions and specific materials entering into well-defined organs and systems of the body? In answering this question, it is necessary to consider the circumstances under which these materials are laid down in the egg, and those found when the substances are utilized in development. The composition of any cell is determined by the operation of its parts. Both experimentally and by the observation of normal processes, it has been determined that the nucleus is highly active in the metabolic functions of the cell. Gland cells in the active state may have the nuclear surface greatly increased, the nucleus lies close to the basement membrane, through which the food enters, and the specific products of secretion appear near the nucleus. The character of the reaction depends upon the nuclear composition—an egg fertilized by its own kind of sperm shows certain forms of activity and produces eventually other eggs of the same type, but if a foreign sperm is used the reactions are different and the form of egg produced in the hybrid body is unlike the normal. The substances formed in the egg and their distribution are determined, like any other cell characters, by the biparental chromosome control. They are not things apart and independent of the nuclear mechanism, but are definitely controlled by it.

At the time of fertilization the egg offers as its contribution a haploid series of chromosomes, in part coming from its paternal and in part from its maternal lines, and a highly developed cell body built up and prepared under the combined action of the biparental chromosome combination from which the maturation chromosomes were derived. The sperm enters, bringing little beyond a haploid set of chromosomes duplicating in source and character those already present. A new individual is produced and its characters are those of its parents—not merely of the immediate two, but of all those preceding. It cannot be asserted, therefore, that certain extra-nuclear parts (which have been formed through the peculiar activities of a given type of cell, controlled as always by its nucleus) are involved in any unique way with racial as opposed to individual characters, or as evidences of cytoplasmic as opposed to nuclear control. This is not to assert, of course, that the form and composition of the egg do not condition the character of early developmental processes, but it speaks for a recognition of the fact that the cell is a unit resulting from the continuous reproduction of similar units and conditioned in its activities by the nature of its inherited organization. But even in the very early stages of development, the influence of the chromosome is shown in the experiments of Boveri (1895) where enucleated egg fragments were fertilized by foreign sperm, and in the larval skeleton exhibited paternal characters. Recently Tennent (1922) has shown that in the cross between the sea urchins, *Cidaris tribuloides* and *Toxopneustes variegatus*, the sperm may influence so characteristic a feature of development as the time and place of mesenchyme formation.

XV. IN RESPECT TO THE NATURE OF CHARACTERS IN INHERITANCE, THE STRUCTURE AND BEHAVIOR OF THE CHROMOSOMES IN MATURATION AND FERTILIZATION ARE PARALLEL

In preceding divisions of this section the facts regarding the close correspondence between the chromosome mechanism and the exhibition of body characters has been brought out; it will suffice here to consider these together briefly: (1) The egg and sperm are alike in their power to transmit the characteristics of the race and the peculiarities of the individual; the egg and sperm are equivalent only in the number and character of the chromosomes they bring to the new organism (Van Beneden, 1883, and many others). (2) Many characters act as units, being, as such, recognizable in the parents, grandparents, and children; individual chromosomes may be traced through the generations, and are the only recognizable cell elements similarly perpetuating themselves. (3) These unit characters are assorted and distributed by the law of chance; homologous chromosomes exhibit the same relations in maturation and fertilization (Sutton, 1902). (4) Certain characters mark the alternative sexes and divide the members of a species into approximately equal groups; there is a recognizable chromosome whose behavior in maturation and fertilization parallels the distribution of the sex characters (McClung, 1901; Wilson, 1905; Stevens, 1905). (5) The behavior of characters in heredity suggests the arrangement of their factors in a linear order in the chromosomes with fixed relations (Morgan, *et al.*); the chromosomes of the germ cells show at certain periods of their development a linear order of their chromomeres with definite and fixed number and intervals (Wenrich, 1916). (6) The characters of the parents enter into the offspring in fixed linked groups; the number of chromosome pairs corresponds to the number of independently assorting character groups (Morgan and collaborators on *Drosophila*). (7) The groups of characters are of unequal numerical value; the pairs of chromosomes differ in size correspondingly (Morgan, *et al.*, on *Drosophila*). (8) The groups of characters assort and recombine by chance in reproduction; the chromosomes assort by chance in maturation and recombine similarly in fertilization (Carothers, 1913, 1921).

With the clear understanding at all times that the functions of the chromosomes are those of integrated portions of a co-ordinated series of cell parts, it would seem from the parallels just drawn between the activities of the chromosomes and the behavior of characters in heredity that beyond any reasonable question the chromosomes are primary factors in the control of the processes which govern development and organization. Any one of these parallels is highly suggestive; taken together they furnish an unanswerable argument in favor of the chromosome theory of heredity. Indeed, it may be confidently stated that few if any generalizations in biology are more firmly established by such a mass of consistent evidence. Further cytological testimony in support of genetical phenomena is now restricted to a considerable extent by the limitations of optical instruments. So far as we are able to go, the parallel is complete, and in the absence of further visible cytological evidence we are justified in assuming a continuation of the principles so far developed.

Meanwhile more detailed investigations of all cellular phenomena have shown a growing complexity and diversity of cell parts, but this increase in our knowledge has in no way weakened the chromosome theory of heredity. That certain characters are due to specific cytoplasmic structures, such as plastids, is merely evidence of a differential function for these cell parts and does not contradict the evidences of chromosome inheritance. It is necessary in the early stages of any investigation to simplify the problem as much as possible by concentrating upon salient features. This does not at all constitute a denial of other elements of the problem, for which an open-minded attitude should always be entertained, but is a practical measure required by our own mental limitations. To deny or to minimize the value of a consistent body of evidence merely because it is not complete in all details is illogical and unfair. In this discussion it has been thought best to confine the presentation to pertinent facts and conclusions developed, rather than to consume the limited space with the consideration of arguments against the chromosome theory. Until some other theory is developed, more consistent with known facts and fuller in its reach, this theory will stand as our best working hypothesis in a most difficult field.

XVI. IN RESPECT TO THE UNITY OF ORGANIZATION WITHIN NATURAL GROUPS OF ORGANISMS, THE CHROMOSOMES SHOW CORRESPONDING DEFINITENESS AND CONTINUITY

It is a truism that in all our complicated taxonomic system the only concrete identity is the individual organism—groups being merely concepts of relationships. On the other hand, it is clear that there are relations of varying intimacy between groups of individuals, and in those which fall within our experience we note that degree of resemblance is indicative of degree of relationship. We therefore assume that in groups which, because of age, must be beyond continuous human experience the same criterion of relationship must obtain. We are always, therefore, seeking out and evaluating the resemblances between individuals as an index of the relationships of their groups. Thus there has been established the concept of the species as a group of individuals so much alike as to be with difficulty distinguished apart, and physiologically so similar that their germ cells are compatible in the production of new individuals of the same kind. Because of this close resemblance in many cases, the limitations of the group are not well defined and the assignment of an individual to one species or another will depend upon the judgment of the classifier. Again some species are completely sterile when crossed, others may produce individuals which are perfect in every other respect except the power to reproduce, while other crosses may be perfectly fertile. Thus we conclude that species are not equivalent groups, but that they do represent the closest association of individuals, theoretically at any rate, genetically related. Since, as has been shown, reproduction is a property of the germ cells and finally of the chromosomes, it is evident that a correspondence of these throughout the group is to be expected. Extensive investigations on large numbers of species, both plant and animal, have shown that throughout a well-defined group of this character the cells possess a like number of chromosomes. While there are some investigators who deny the specific numerical constancy, and while there are some cases of unquestioned variation (most of which are readily explainable), there is little doubt that there is a strict relation between a definite organization of the chromosome complex and the form of body organization which is recognized as specific in value. The basis for this conclusion has been more fully discussed elsewhere in this section.

If this relation between chromosome organization and body form obtains for the species it is not unreasonable to expect that there should be cases in which it would prevail throughout groups of species, whatever these might be called. The a priori argument for this, stated cytologically, would be: Since the chromosomes reproduce themselves in each mitosis, and are thus the same in all the cells of an individual, since the individuals of the species are genetically related to each other through descent from common ancestors and thus have the same chromosome complex by direct inheritance, in species structurally much alike, having, therefore, ancestors only one more step remote, the chromosome group would trace directly, though more distantly, and thus be numerically the same. A like argument would hold through more inclusive groups, the only premises being the individual reproduction of the chromosomes (an observed phenomenon) and the continued organization of the chromosomes (assumed).

An extensive study of the family of short-horned grasshoppers, *Acrididae*, has been made by McClung and his students (1900–1923). This has included almost all the genera of North America, some from Mexico, and a few from the islands off the northern coast of South America. With a few exceptions, all susceptible of reasonable explanation, this great and well-defined group of insects shows in the male animal a diploid complex of twenty-three chromosomes and the female one of twenty-four. Constancy in number of chromosomes, taken in connection with the observed individual reproduction of each chromosome in mitosis, can mean only one thing, that is, the homology of the series and its genetic relations. But we find not only an identical numerical series in each species but a comparable order of sizes ranging from a small element to one many times its length at the opposite end of the scale. Finally, throughout this entire range of species with its countless multitudes of individuals, one particular chromosome, the accessory chromosome, is always distinguishable from the others by definite structural and functional characteristics. It is of much additional significance to note here that this chromosome may be recognized, not only in the family *Acrididae*, but in all insects and a great many other animals. That is to say, the chromosome determinant for sex differences is found in a measure coextensive with the existence of sex itself.

In considering the existence of a material substratum for the exhibition and control of hereditary processes, the significance of one recognizable chromosome of the series, demonstrably associated with the development in each individual of a particular series of characters, cannot be overestimated. Sex differences mark every cell in the body of male and female, and here is an element which so modifies the activities of the other chromosomes as to incline development to the male side under one condition and to the female under another. It would be a mistake to stop with merely a statement of the parallel in this form; we have much to learn regarding the operation of the chromosome mechanism by noting the variations in the exhibition of sex characters and the differences in the behavior of the associated chromosomes. Sex, being the expression of a common series of characters in two aspects, may be expected to show various gradations of contrast. In the Orthoptera, as in most insects, there is the sharpest separation between the sexes, the female always being the larger and sometimes very much so, while the so-called secondary sex characters are well marked. There is no indication of hormone action, each cell being definitely and permanently of male or female character and independently so, apparently. The chromosome conditions are of the presence or absence form—one, or two, accessory chromosomes: the sex characters are of similar distinction. In other insects, e.g., the Hemiptera (Wilson, 1905–12), there is a Y-chromosome, but this is confined to the male line and has little or no influence, so far determined. Gradation is absent and there is no hormone control.

On the other hand, there are cases where there is great lability in the expression of sex differences. In *Crepidula*, Gould (1917) showed that the inclination one way or the other depended upon the size of neighboring individuals; in *Bonellia* Baltzer (1914) found that attachment of the young to the proboscis of the female was necessary for the production of males, the individuals otherwise becoming females; castration of crabs by *Sacculina* changes the body form to that of the female (Smith, 1910, 1911); Goldschmidt (1920) produces a great range of intersexes in *Lymantria* by crossing different races; Crew (1923) finds that destruction of the ovary of functioning hens by disease may lead to the transformation of the individual into a functional male. These and many other instances show that absolute fixity in the expression of sex characters may be lacking in some forms. Are we to assume from this that sex is determined in a different way in such cases from what it is in the Orthoptera, or should we expect to find a variation in the operation of a common mechanism? If sex is a characteristic of all higher organisms, if it consists in an alternative expression of a common series of characters, if its mechanism of determination is a part of that for the determination of all characters, then it must be a part of this system and subject to the laws of the system. We have difficulty in accounting for sex differences only when we consider them something apart and distinct from the rest of the organism. Sex is indeed an integral part of the individual and must be explained in terms of the entire complex. Intersexes, gynandromorphs, hermaphrodites, and other manifestations of unusual sexual conditions are, therefore, not new things to be recognized, but modifications of the bisexual plan, whose interpretation will aid us in understanding the operation of the whole method of character development and differentiation. Since, therefore, we find in many cases a definite association between the appearance and behavior of a certain recognizable chromosome and the development of sex characters we are warranted in regarding their relation as that of cause and effect (in view of our knowledge of cell processes), operating as a differential in a co-ordinated complex which includes the other chromosomes, the cytosome, and the environment. In this respect it is like all the other chromosomes, and so we draw the conclusion that since there are, throughout the *Acrididae*, always a constant series of chromosomes, comparable in many ways, and since we can associate one member of the complex with a definite function, we may assume similar homologies for the others.

XVII. WHILE THE RELATION BETWEEN CHROMOSOME STRUCTURE AND BEHAVIOR AND CHARACTER INHERITANCE IS MEASURABLY DETERMINABLE, THE NATURE OF THE PROCESSES WHICH CONTROL DEVELOPMENT REMAINS COMPARATIVELY UNKNOWN

If it be asked: How do the chromosomes control development? the reply must be a confession of ignorance. An answer here waits upon a solution of the more general problem of how protoplasm functions and especially upon an understanding of the co-ordinated processes of its unit manifestation—the cell. There is much evidence to show that there is a dual nature to the cell, its body being in intimate contact with the environment, taking in food, giving off waste, forming contacts, executing movements, etc., while the nucleus, more remote from these conditions, reacts in response to the varied requirements of the entire unit, governing the rate, degree, and time of its processes. Apparently also the type of reaction between nucleus and cytosome is that characteristic of osmotic processes. There is obviously a difference in density between these two cell regions, and in very active secreting cells, the nuclear surface is greatly increased so as to afford a maximum of contact with the cytosome. In many instances the production of materials in the cytoplasm can be noted next to the nucleus.

These features of increased nuclear surface and the production of specific cytoplasmic substances are particularly noticeable in the germ cells, and offer suggestions regarding the possible differential action of the chromosomes. As was described by Sutton (1900) (Fig. 9) in *Brachystola*, the chromosomes of the spermatogonia during their rapid multiplication become highly vesicular, each forming, as it were, a little nucleus of its own. Under these circumstances the cytoplasmic materials are quickly used up and after some eight or ten successive mitoses, the final generation of spermatogonia consist almost entirely of nuclei. During this period the chromosomes have been present in the diploid number, and have been functioning under the same conditions as the somatic cells. In the last generation of the spermatogonia, therefore, it may be said that the chromosomes have practically exhausted the cytoplasmic environment created while the homologous chromosomes have been operating as separate nuclear bodies (Fig. 40). These relations are here somewhat suddenly ended by synapsis of the homologous chromosomes—a process followed by exactly the opposite series of the cellular activities to those of the spermatogonia. These cells, now the first spermatocytes, do not divide for a long while but increase greatly in size;

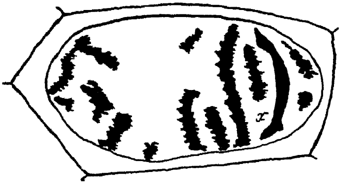


FIG. 40.—Late spermatogonium of *Mecostethus* with nucleus and cytosome much reduced in size. The chromosomes extend directly across the nucleus, the accessory chromosome, *x*, condensed and homogeneous (McClung).

and the cytosome, which was reduced to a small fraction of the dimensions of the nucleus, grows again to relatively large proportions (Fig. 39). It may be said, indeed, that the cell is rebuilt under the activities of the chromosomes while in the synapsed condition. If we review the process of fertilization we find that it is first a union of two cell bodies and then a union of their nuclei. Following upon this, occur all that complicated series of processes which characterize differentiation and development, during which the germ cells are set apart and remain unchanged. In this connection it will be recalled that in *Ascaris megalocephala* such distinction between somatic and body germ cells starts in the two-cell stage, and is marked by a fragmentation of the chromosomes in the one case and by a *complete preservation of their organization in the other*

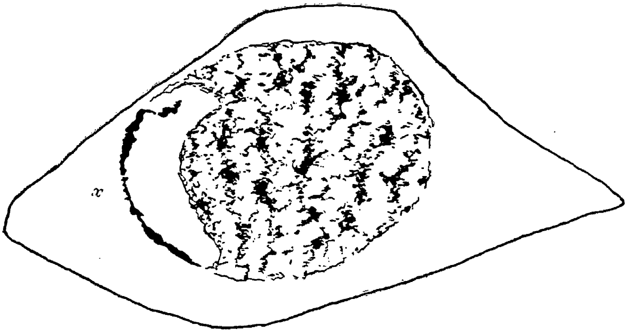


FIG. 41.—First spermatocyte of *Mecostethus* during the growth period. Both nucleus and cytosome much enlarged as compared with their dimensions at the beginning of this period, but the latter disproportionately so. Cf. Fig. 40. The chromosomes at this time are much diffused. The accessory chromosome, *x*, lies in a large vesicle of its own (McClung).

(Fig. 20). A union of cells and nuclei, with differentiation of the chromosomes in *Ascaris*, marked the steps involved in the establishment of a new animal body—the union of homologous chromosome appears to be the first step in preparation for the succeeding generation—a single cell which is at once somatic and germ in character.

The differential action of the accessory chromosome in *Brachystola* and other Orthoptera is suggestive. Although only one of the twenty-three chromosomes, it often has a vesicle which is of unusual size (Fig. 41). If an extent of surface is any measure of the degree of metabolic exchange, the accessory chromosome is exerting a disproportionate effect, and might be said to be determining the differential character of the cell during this period. Since this differential is finally that between the sperm on one side and the egg on the other its specific determinative action is, therefore, concerned in shaping the development of the specialized cell which gives rise to the sperm (McClung, 1901). In the ovum the accessory chromosome is paired with another and, so far as our limited observations go, there is lacking such a differential behavior. The growth of the cell here is very much greater, at least in the storage of food material. Stating the matter purely objectively, therefore, we would say that in the sperm-producing cells, added to a diploid series of chromosomes is a single accessory chromosome, which, during certain periods, is disproportionately active judged by the relatively large surface exposed for metabolic exchange; while in the case of the egg, conditions being the same except for the presence of two accessory chromosomes, there is no such evidence of differential activity. In the one case, growth is limited, and the resulting cells are the highly specialized sperms; in the other growth is excessive and the food-laden egg is the result. Since the accessory chromosome constitutes the only apparent structural differential in the two cases, it might be said that, in the sperm-producing cells, it operates as an inhibitor of growth, or, on the contrary, that it directs cellular operations toward the production of very specialized structures. As a matter of fact, both things happen, but how is as much an uncertainty as the whole problem of growth.

The case again may be presented somewhat in this way: Under a given and relatively fixed environment, at a certain period in the history of an individual, its germ cells, in the form of eggs, prepare for reproduction. In this process the cells of the body nourish the eggs and supply them with materials for growth of a highly specific character—these materials being produced by cells whose functions are determined by a duplex series of chromosomes equally derived from the male and female parents. The materials thus derived are taken into the egg and there elaborated and organized into the form characteristic of the egg of the species—again under the influence of the same duplex series of chromosomes as is present in the body cells. The original duplex series of chromosomes, having thus operated in the organization of the egg, is, during maturation, reduced to a simplex condition, not by the elimination of either the paternal or maternal group, but by a chance selection between the various homologues of the two series. In this condition the egg may possibly, either under natural or experimental conditions, undergo development and produce a perfect organism of its own kind. Its chromosome mechanism is complete, and adapted to the cellular conditions which it helped to create and to the environment in which the whole is placed. Commonly, however, before development occurs, a sperm, bearing a simplex series of chromosomes, comparable in almost every way to that of the egg and representing also a chance assortment of a preceding maternal and paternal series, enters and takes the place of the one which has been thrust out by the egg. *This process does not in any way change the relative functions of the nucleus and cytosome; it merely changes the members of a duplex series of chromosomes by bringing in half the set from another individual.* Under other circumstances this restoration may take place by the re-entrance of a half-group that has been thrust out in a polar body. The egg at the time of fertilization is a cell organized, under the conditions of all cellular growth, by the interactions of nucleus, cytosome, and environment, but in this case has disproportionately built up the cytosome in preparation for a series of rapid cell divisions without intervening periods of growth. The course of these resulting processes is, therefore, fixed in advance for some time, *not by the cytosome alone*, it must be observed, for this is merely the register of the operations of the cell in its peculiar environment. Under the conditions of the male body, at a similar period, the cytosome does not undergo this unusual growth, but pursues a contrary course; herein is the differential character of male and female in relation to their germ cells. The history of the chromosomes, however, is essentially the same. Neither the half-set of chromosomes in the sperm nor the stimulus of its entrance into the egg is absolutely required for development, as parthenogenesis shows, but they are commonly elements of sexual reproduction.

Now in species matings, fertilization merely slips in a set of chromosomes to take the place of a corresponding set which has been extruded by the egg, the old egg nucleus, as such, being at the same time destroyed. The egg pronucleus, which then forms, and the sperm pronucleus unite to produce the cleavage nucleus, and cell division starts off normally, but with such relatively minor changes as may be determined by the altered combination of chromosomes. That the processes of maturation and fertilization result in this diversity of expressing a common series of characters is very evident from an investigation of the offspring of any pair of parents, no two of which are nearly alike except they come from the same fertilized egg. In cases of hybrid matings, however, when the set of chromosomes introduced by the sperm does not fall accurately into the vacant places left by the removal of a haploid series in maturation, conditions of development are different. Sometimes they take no part whatever in hereditary processes, the sperm serving merely as a stimulus, just as chemicals or mechanical agents do, in which event the characters are purely maternal (Herbst, 1909, 1912; Balzer, 1910; Godlewski, 1906); again there may be a slight participation of the paternal chromosomes (Tennent, 1912, 1922); finally, they may apparently perform normally in the processes of the cell (Pinney, 1918, 1922).

Crossing experiments have been used to test the relative influence of the parents in heredity, and in those cases where genetic results are the criteria, it has been found that it is a matter of indifference whether the sperm or the egg carries the determiner (*Drosophila* studies by Morgan, *et al.*, Castle on various mammals, etc.). Curiously enough, because the earliest stages of development are not at once visibly modified in hybrid crosses the conclusion has been drawn that the paternal chromosomes are without influence or that their influence appears later or only in minor matters. J. Loeb, noting the high degree of visible organization in the egg, carries this view to its logical conclusion, and denies the nucleus any profound part in heredity in these words: "This may mean that the protoplasm of the egg is the future embryo, while the chromosomes of both egg and sperm nuclei furnish only the individual characters" (Loeb, 1916, p. 70). A sufficient answer to such a statement would seem to be the citation of the control through the chromosome mechanism in *Drosophila* of the presence or absence of eyes or wings or other essential parts of the body, or even of the balance in functions which makes existence possible. Since it has been experimentally determined that no development can occur without a nucleus, it is hard to conceive any set of conditions which would permit the early stages of development in its presence and without its influence. Logically, also, it is difficult to conceive the nucleus operating throughout development, and being necessary thereto, and having effects which are only individual or superficial, or late in appearance. In other words, if the cell is the structural and functional unit which all our studies tell us it is, the operation of its parts must be not only co-ordinated but continuous, and any effects produced must result from the performance of the entire series and not from parts. This, of course, does not mean that there is no differentiation of function, as some assume, or that the "cell as a whole" is anything but the co-ordinated sum of its parts; it does signify that in a mechanism of differentiated, co-operative members they must all work together all the time. Fortunately, also, we have experimental proof of this continuity of action and the early influence of the paternal chromosomes.

As indicative of the early participation of the sperm chromosomes in development the observations of Tennent (1920) upon inter-ordinal crosses between *Arbacia* and *Moira* are suggestive. In straight-fertilized eggs, there are lacking certain rods and granules in the cytosome which are strongly present in the crosses. Something brought in by the foreign sperm produces an effect upon the cytosome not apparent when the species sperm is used. It may be assumed here either that this effect is entirely new or that it is an unusual manifestation of a common reaction between nucleus and cytosome. Tennent is inclined to the latter opinion, and Miss Hibbard (1922), working under his direction, found that, in crosses between *Toxopneustes* and *Hipponoë* where development proceeds normally, these substances appear and later disappear, apparently being used in the cellular processes. The reasonable explanation would seem to be that the foreign chromosomes are merely indicating by their unusual reaction with a strange cytoplasm a normal process of nuclear-cytoplasmic exchange. Thus at the very first cell division there are indications of the influence of the nucleus in development.

The work of Tennent upon hybridization in echinoderms is very suggestive in regard to the relative parts played by nucleus, cytosome, and environment. He found (1910) that in a generic cross between *Hipponoë* ♂ and *Toxopneustes* ♀ the plutei were dominantly paternal. Here, although the *Toxopneustes* egg supplied the entire cytoplasm and half of the chromosomes, the characters were largely determined, even in this early stage of development, by the sperm chromosomes. When a reciprocal cross was made with the *Hipponoë* chromosomes coming from the mother, the plutei were again of the *Hipponoë* type. In other words, the *Hipponoë* chromosomes, no matter what their source, prevailed in their influence and shaped the processes which were involved in the successive cell divisions, cell reactions, and cell arrangements leading to the formation of a pluteus. In a later, detailed study of the chromosome conditions in these crosses (1912), Tennent found that in the case of the *Toxopneustes* egg fertilized by the *Hipponoë* sperm there was no chromosome elimination so that in this instance of paternal dominance in embryo characters, the effect of the foreign chromosome was exerted in the presence of the female complex. In the reciprocal cross, however, the *Toxopneustes* chromosomes, brought in with the sperm, were gradually eliminated in successive mitoses until after the sixteen-, or later, cell stage there remained only what were presumably the maternal chromosomes. This would seem to be a clear demonstration of the part played by the chromosome in those stages of development which Loeb and others regard as laying down the structures characteristic of the species, genus, and other more inclusive groups.

Although there is here nothing to indicate the part of the egg cytosome in the developmental processes, it is entirely obvious that the pluteus is formed out of the materials supplied by it. Tennent's results show that the pattern or control of the movements which mold these materials is furnished in large part by the *Hipponoë* chromosomes.

But this is not all the story of these hybridizations—it represents only that part of it where the environmental conditions remained at a given level of H-ion concentration. By adding an acid to the sea water in which fertilization and development of *Toxopneustes* eggs fertilized by *Hipponoë* sperm occurred, the characters of the plutei could be swung over to the *Toxopneustes* side. Internal conditions of the egg remained as before at the time of fertilization, but the environment was changed and a different result followed. The cytological conditions following from this environmental change were not determined to the satisfaction of Tennent, but in his opinion elimination of *Hipponoë* chromosome was indicated by the lessened total chromosome number in the cells. These experiments indicate clearly that in accounting for any developmental result, it is necessary to consider every element, because each plays a part.

It is, indeed, quite futile to attempt a separation of the parts of the cell functionally and to assign to one a particular type of character development and to another portion other characters. Whatever there is in the cell it is there because of the co-ordinated activities of the cell as a unit. If there are particular substances in the egg which go to the formation of definite regions or organs they are there because, under the indispensable influence of the nucleus, they have been made a part of the cell through its peculiar metabolic processes. The form and arrangement of the resulting cells are determined, not by the mere presence of these differentiated materials, but, in each case, by the nature of the interaction between a nucleus and certain combinations of cytoplasmic substances in which it lies and with which it works. The thing of greater significance to note is that while there is no demonstrable continuity between these types of extra-nuclear materials, which, indeed, vary in amount and character in successive cell generations, there is a complete continuity between each member of the chromosome complex which individually reproduces itself at each cell division.

Of all the remarkable characteristics of living things, one of the most astonishing is the persistence of a relative uniformity in specific characters over periods of time extending into the millions of years and involving innumerable individual repetitions. As a basis for this continuity of form there must be a mechanism of corresponding fixity: nowhere is there such a cellular basis for uniformity except in the chromosomes. Cytoplasmic materials are constantly differing from one cell generation to another and from tissue to tissue. As Minot pointed out in his theory of cytomorphosis, the cytoplasm registers the stage of differentiation and ultimately becomes so highly specialized as to fix the cell as a given type. But always the group of chromosomes or its materials are recognizable. In some organisms, even after this definitive organization is accomplished, a disturbance of it by injury will lead to regeneration, in which case the cells undergo a series of regressive changes before they can begin again their specialization. By a change in cell environment intracellular relations are altered, the form and structure of the cytosome becomes dedifferentiated, and the nucleus operates under altered conditions, but apparently without structural change so far as the chromosomes are concerned. It may be suggested that this is proving too much—that if the chromosomes are always the same in their characteristics they cannot be determiners of differences. Such a position might be tenable if the nuclear apparatus were an independent thing, but instead it is a part of the cell, secondarily in contact with external conditions and co-ordinated with the cytosome which has the primary relation with the environment. Any action of the cell means a response or adaptation to this environment, and is strictly limited by it. The cytosome naturally is the first to feel a change, and in turn presents an altered relation to the nucleus. A readjustment occurs between these elements until a state of relative stability is reached. No continued response could be made without a nucleus in the cell, and the nature of it when made is determined by the nucleus. In this action, however, there are always the three necessary elements—nucleus, cytosome, and environment. Change any one and the result is altered, but this does not mean that they play the same parts. To assert that the chromosomes exert a guiding or directing action does not imply that they accomplish the whole effect, but is merely an effort to assign them their place in the series of reactions involved.

Reference has already been made to the relation between the accessory chromosomes and sex characters, but it may perhaps help in gaining an understanding of the operation of the cellular mechanism if we go a little farther into a consideration of this relation. Sex, physiologically, is a division of labor in reproduction, between two kinds of individuals in the species, one of which is preponderatingly anabolic, the other katabolic. These characteristics in the rate and degree of metabolism are expressed in every activity of the body, and are not confined merely to the reproductive functions, although they are often more strongly manifest there where their primary activity lies. While there are various modifications in the expression of these physiological differences between the sexes they are preserved in their general character throughout almost the entire range of organisms. Because the various aggregates of cells constituting the organs of the body exhibit constant differences in the performance of a common series of processes in the two sexes, we must assume that each cell of the aggregate is similarly marked as between male and female. This assumption is practically demonstrated in mosaic gynandromorphs, where very small groups of cells showing respectively male and female characters lie side by side in the same body. Like other bodily functions, therefore, sex is a matter of cell activity, and must ultimately be explained in terms of cell performance.

Difference of behavior implies corresponding difference of structure. Such is often strikingly manifest in the bodies of male and female, but, until the beginning of the present century, the cellular basis for the differential behavior of a common series of functions was not understood. From an extensive series of investigations (McClung, 1901; Wilson, 1905; Stevens, 1905; and many later workers), it appears that commonly there is one chromosome which is directly and demonstrably associated with the differences which mark the sexes. Because of this definite association between a given chromosome and the exhibition of recognizable body characters, we have presented a better opportunity for the study of the method of operation of the hereditary mechanism.

First, it should be observed that what we have to explain in accounting for structural differences between male and female is not anything unique in either, but the differential development of a common series of characters which bear in many ways a lock-and-key relation to each other in the two sexes. In many cases this means the establishment, during development, of an apparently undifferentiated rudiment which later develops in one direction, if the individual be a female, and in an opposite direction, if a male. It is to be noted that there is not only the question of differences between the sexes, but of differences which are reciprocal. Further, it is to be remarked that development is not always of a plus character in one sex and a minus in the other, but that there is a mixture of these in both. This means, of course, that certain regions or organs or cell aggregates show an excessive growth in one sex as compared with the other, while in another region a reverse relation would obtain. Since it can be demonstrated that in the fertilized egg producing a male there is commonly a single accessory chromosome[1] while in one giving rise to a female there are two, the ultimate structural analysis means that in each cell of the male we have this chromosome difference as compared with the female. Organs and tissues differ sexually, therefore, because their cells differ. We must regard these differential chromosomes as modifying the functions of the entire complex with which they are associated, not as contributing anything peculiar to the organism, so far as their sex-determining function goes. That they also bear determiners for characters not peculiar to sex has been experimentally established in *Drosophila*. In respect to sex determination they are, therefore, accessory to the remaining chromosomes serving to modify their activities.

So far we have little observational evidence to indicate how this differential effect is exerted. As is explained elsewhere in this section, it appears that in the formation of the paternal germ cells the accessory chromosome proceeds in its changes at a different rate and degree from the euchromosomes. There are indications from the experimental embryological work of Stockard (1921) and others that if an organ is prevented from developing at its normal time, the rest of the body continues on its course and the delayed part never has opportunity to recover its lost ground. That is to say, time is an element in development, and once it has passed cannot be recovered. If there is this time element, in the operation of cell aggregates, it must also obtain in the inner processes of cells. What evidence we have of the behavior of the accessory chromosomes in the female does not indicate such differential activities, although these elements may sometimes be recognized by structural peculiarities. They seem to act synchronously with the euchromosomes.

It has been found by Bridges (1913) that the source of the sex-determining elements is immaterial—one, whether derived from paternal or maternal source, operating to produce male characteristics. He has also reported a graded series of intersexes corresponding to like grades in the ratios between the numbers of sex chromosomes and euchromosomes. Recently Schrader and Sturtevant (1923), adapting Goldschmidt's formula for *Lymantria*, have suggested that it is not the numerical ratio between the euchromosomes and the *X* chromosomes which determines sex, but antagonistic action between the euchromosomes representing maleness and the *X* chromosomes representing femaleness. These explanations of the operation of the sex-determining mechanism are formal in nature, dealing with factors or genes, and carry the implication that sex is something separate and apart from other characters of the body. As has just been suggested, however, sex is an inherent part of the organism, and male and female represent merely two aspects or manifestations of a common series of structures and functions. What we have to explain accordingly is how development proceeds in one individual so as to give these common characteristics the stamp of maleness and in another that of femaleness. Because the result is a question of degree the suggestion lies near at hand that degree of activity of the operating cause is responsible. But since the same mechanism is present in all the cells of the body and some of these, in the same individual, show what is evidently a plus action while others evidence a minus activity, how are we to account for these indications of variable action? So far the only visible evidence of difference in the activity of the sex chromosomes is that just described in which, at the period of germ-cell differentiation in the Orthoptera, the accessory chromosome shows excessive and non-synchronous action. A result, or at least a concomitant, of this is the production of the highly differentiated sperms. If we assume that in the development of the body similar differential action occurs, now stronger, now weaker, we can gain some conception of the differential effects. This is, of course, only another aspect of the general problem of differentiation, for with the same chromosome mechanism in all the cells derived from the original complex of the one-celled egg, the infinite varieties of bodily structures are produced. What appears in sexual differences is, in addition, an alternative aspect to each of these conditions. Because, however, this relatively simple but all pervasive, influence is associated with definitely recognizable chromosomes it is perhaps more possible to determine causal relations here than in the case of the other chromosomes—the assumption being that the general relation of all chromosomes to the cytosome is similar. Evidence from genetical experiments is convincing that the chromosomes are, qualitatively, individually different. Cytological evidence of variation in the time and degree of their action is less available. What has been described for the accessory chromosome is in some degree also to be noted for some of the euchromosomes during orthopteran spermatogenesis. The precocious chromosomes described by Wenrich for *Phrynotettix* are an example of this. Observations upon the behavior of particular chromosomes during development are unfortunately lacking, but studies upon the abnormal conditions in hybrids show clear and striking differences in the reactions of the species and foreign sperm, as well as individual variations in the latter. From all these considerations, therefore, we are inclined to say, as our best judgment in a matter of extreme difficulty, that the differential action of a common series of chromosomes in all the cells of the body is due to variation in the rate, degree, and time of their individual reactions, and that, because during development at every cell division there is a segregation of materials, they come to lie, in each case, in a differently organized cytosome. In turn the cytosome thus meets a different environment, at the very first division being proportionately much reduced in its contact with the physical environment through the intervention of its sister-cell, and this continues progressively until direct contact with external conditions is greatly reduced or entirely lacking for most cells of the body.

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[1] It is immaterial to the argument that reverse relations occur in some groups.

It is difficult to imagine a simple indication of how these interactions between nucleus, cytosome, and environment might operate in so complicated a matter as differentiation, but if it might be supposed that a given effect is produced because the action of a certain chromomere, in relation to all the other nuclear elements, takes place by its intereaction with the cytosome only in the presence of light, it is obvious that this result would not occur in those cells entirely shielded from the light by others and to a proportionate degree only in those partly protected. If this action should be due directly to the oxidation of materials in the cytosome, and the rate of cell division be proportionate to oxidation, then cells exposed to light would divide more rapidly than those shaded, and would thus overgrow them more and more. Further, if gravity were free to orient the organism in its early development so as to favor this light action and its resultants in certain groups of cells, then these would be the ones to proliferate most rapidly and prevail at this period of growth. Such a supposititious case might be continued farther, taking into consideration more of the known factors of development, but at best it would be entirely inadequate and fragmentary. It may serve, however, to indicate in a rough way the interdependence of the multitude of factors in operation at any given time, and to explain how certain cells might behave differently from their neighbors, although all were provided with identical chromosome complexes.

In a problem of such infinite complexity no complete and final explanation is possible. Our only method of attack is to analyze, so far as we can, the operation of the various factors involved. Assigning to one element of the series its appropriate function does not involve the implication that it is the only factor. To state that one member of the series is primary, initiatory, directive, or regulatory does not lessen the significance or necessity of the others. Studies upon the structure and behavior of the chromosomes in relation to known facts of inheritance have established certain correlations that apparently stand in the relation of cause and effect. These correlations have been extended and systematized until they now constitute the largest contribution yet made toward our knowledge of heredity. It is recognized, however, that the advance thus far is very small and in only one field. A formal explanation according to a factorial system accounts, in a very large measure, for the mechanism of transmission from parents to offspring of their particular expression of specific characters; we have as yet little understanding of how these factors, in relation to the cell and its environment, shape the course of development. The chromosome theory as it stands is logical, consistent, and generally applicable to both plants and animals. Admittedly incomplete, it yet stands as one of the highest achievements in biology and offers the most promising guide to further advances.

XVIII. BIBLIOGRAPHY[1]

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[1] The literature in this field is so extensive that it is impossible in the space available to give more than a fraction of the references. Those here included are confined almost entirely to the cytological aspect of the subject. Only such as have general interest or bear directly upon the discussion are listed. An asterisk (*) indicates general works.

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SECTION XI

MENDELIAN HEREDITY IN RELATION TO CYTOLOGY

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MENDELIAN HEREDITY IN RELATION TO CYTOLOGY

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The extraordinary advance in our knowledge relating to the germ cells that took place during the last quarter of the last century prepared the way for a cytological interpretation of Mendel's principles immediately after their recognition in 1900. Within two years after the rediscovery of Mendel's paper in 1900, the application of the results of cytology to Mendel's two laws was pointed out (Sutton, 1902). Since that time the significance of this relation has become more and more apparent with every new advance, both in genetics and in cytology.

The behavior of the chromosomes in the maturation of egg and spermatozoön not only furnishes an exact parallel to the genetic behavior of the postulated Mendelian factors, but crucial situations have also been met with (such as non-disjunction, the elimination or addition of specific chromosomes) that have furnished very strong confirmation of the view that the chromosomes are the bearers of the genetic elements. Other genetic phenomena such as linkage and crossing-over, that were unknown to Mendel, have also been brought into close relationship with chromosome behavior. From a consideration of linkage and crossing-over, still further possibilities have presented themselves that promise to throw light on the configuration of the hereditary elements borne by the chromosomes. The analysis of crossing-over in relation to the chromosomes carries us into a region far beyond the visible limits of even the most powerful magnifications that are possible today. In this respect, genetics has proved a more refined instrument in analyzing the constitution of the germinal material than direct observation of the germ cells themselves, and while this advance may appear more theoretical than the conclusions based on observations of the cell, this need not mean that it is less reliable.

I. MENDELIAN CHARACTERS AND THE POSTULATED ELEMENTS IN THE GERM MATERIAL

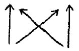
The two independent processes that are included in Mendel's principles may be referred to as (1) the law of segregation of the members of each pair of factors, and (2) the law of independent assortment of the pairs of factors. It follows from the second law that the germ material is composed of independent units, or, in other words, heredity deals in its theoretical aspects with discrete units, not with wholes; yet, paradoxical as it may appear, the characters that are inherited must be supposed to be due to the interaction of a large number of hereditary units. In other words, the *character* is to be considered as a whole rather than as a unit. Since the establishment of Mendel's second law rests on the validity of the first law, the latter must be dealt with first.

A concrete case will serve to illustrate Mendel's first law. If a tall pea is crossed to a dwarf pea, the offspring (F_1) are all tall. If these tall, F_1, peas self-fertilize (or are bred to each other) the offspring (F_2) are tall and dwarf (like the two grandparents) in the ratio of three talls to one dwarf. If these F_2 peas are allowed once more to self-fertilize it is found that all the dwarfs give rise only to dwarfs (F_3). This means, of course, that all the dwarf grandchildren (F_2) are pure for the dwarf character, although both their parents were tall, and only one of their grandparents was dwarf. On the other hand, when F_2 talls are allowed to self-fertilize, it is found that while some of them produce only tall offspring (F_3), others produce both tall and dwarf offspring (F_3) in the ratio of 3 : 1, which is the same ratio as that given by the first-generation peas (F_1).

Mendel pointed out that these numerical relations could be understood if we assume that the tall pea supplies an element for tallness and the dwarf pea an element for dwarfness. These are brought together in the hybrid (heterozygous) peas of the first generation (F_1). If now these elements separate in the germ cells so that half of the germ cells contain the element for tallness only, and the other half of the germ cells contain the element for dwarfness, then chance union of any pollen grain with any egg is expected to produce F_2 offspring of three kinds; for when a dwarf-bearing pollen grain meets a dwarf-bearing egg, the combination will be pure for dwarf, and when a tall-bearing pollen grain meets with a tall-bearing egg, the combination will be pure for tall; but when a tall-bearing pollen grain meets a dwarf-bearing egg, or else a dwarf-bearing pollen grain meets with a tall-bearing egg, either combination gives an individual that is hybrid, i.e., it contains an element of each kind. Such individuals will be tall; for, as shown in the F_1 hybrids, the combination of dwarf and tall gives tall. The expectation in the second generation (F_2), therefore, is one pure tall to two hybrid tall to one pure dwarf. In other words, in F_2 there are three talls to one dwarf.

Eggs ....tall and dwarf

Gametes of F_1



Sperm........tall and dwarf

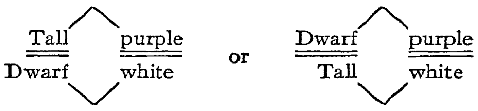
F_2 Peas: 1, tall tall; 2, tall dwarf; 1, dwarf dwarf.

It will be observed that each individual of this second generation has a pair of the elements under consideration which may be tall tall, or tall dwarf, or dwarf dwarf. Each individual may be said to be duplex, consisting of pairs of elements that separate (segregate) at some time before the germ cells reach their full maturity. These paired elements that segregate are called allelomorphs.

The elements of each pair may be alike or different. This duality is not something peculiar to Mendelian hybrids, it is true of pure forms as well, so that the two parents in the foregoing cases must also be treated as duplex, but their ripe germ cells, like those of hybrids, contain only one member of each pair. When the members of any pair are alike, the individual is homozygous for that pair, when they are different, heterozygous. The elements that make up the allelomorphic pairs are called genes.

When only a single difference, such as tallness and dwarfness, is involved in a cross, the results could be equally well accounted for on the assumption that the whole of the germ material is involved; for, obviously, the outcome would be the same if the whole tall-pea complex that went in from one parent separated in the germ cells of the hybrid from the whole short-pea complex. On the other hand, when two or more pairs of characters are involved in the same cross such a possible interpretation no longer holds. This may be shown by another example, which illustrates also Mendel's second law.

If a tall pea with purple flowers is crossed to a dwarf pea with white flowers, the offspring (F_1) are all tall with purple flowers. If these (F_1) self-fertilize, they produce four kinds of offspring in the ratio of nine tall purple to three tall white to three dwarf purple to one dwarf white. These results are explicable if in the germ cells of the F_1 hybrid, tall and dwarf behave as one pair of allelomorphs, and purple and white as another pair, and if the members of each pair behave independently of the members of the other pair. This distribution of the factors in the F_1 hybrid may be represented graphically as follows:



As a result of this independent sorting out of the members of each pair, there will be formed four and only four kinds of gametes, namely, (1) tall purple, (2) dwarf white, (3) dwarf purple, (4) tall white. Chance fertilization of any one of the four kinds of egg cells by any one of the four kinds of pollen grains gives sixteen recombinations. If these F_2 recombinations are sorted out and named according to the dominant characters, when they carry such, and according to the recessive characters (when they are homozygous for these, i.e., when the dominant gene is absent) there will be found to be only four kinds of peas in the ratios of 9:3:3:1. The sixteen recombinations are shown in the diagram (p. 696). Mendel also proved that when three pairs of characters are present in the same cross, the members of each pair behave independently, producing eight kinds of gametes. These by chance recombination give sixty-four individuals of eight kinds in the F_2 generation in the ratio of 27:9:9:3:9:3:3:1. His results, as well as those obtained later by many students of genetics, show that the rule of independent assortment holds for as many pairs of allelomorphs as enter the cross, *provided each pair belongs to a different linkage group.* The explanation of this restriction of Mendel's second law will be given later.

The occurrence of free assortment makes it necessary to assume that the germ material consists of independent units, since the results cannot be accounted for on the assumption that whole germs that enter from the two parents separate as such, when the gametes are formed in the hybrid. Thus in the illustration above the characters tall and purple went in from one parent, and dwarf and white from the other parent, but some of the F_2 offspring are tall and white, and others are dwarf and purple. The same result holds when more than two characters enter the cross together, hence, in a sense, the characters that enter from the two parents are found disassociated in the second generation. This can only mean that those elements in the germ cells standing for the characters of the individual behave as independent units or elements. It follows, in so far as this occurs, that there is free assortment of the pairs of genes in the germ cells of the hybrid at the time when the number of genes is reduced to half. There is some further evidence that this sorting out does not take place before the final or maturation division of the germ cells, and if this can be established, it may be said that *the free assortment of the members of different pairs of genes takes place at the time of maturation.*

	EGGS			
POLLEN	Tall purple	Dwarf white	Tall white	Dwarf purple
Tall purple	Tall purple Tall purple (9)	Dwarf white Tall purple (9)	Tall white Tall purple (9)	Dwarf purple Tall purple (9)
Dwarf white	Tall purple Dwarf white (9)	Dwarf white Dwarf white (1)	Tall white Dwarf white (3*a*)	Dwarf purple Dwarf white (3*b*)
Tall white	Tall purple Tall white (9)	Dwarf white Tall white (3*a*)	Tall white Tall white (3*a*)	Dwarf purple Tall white (9)
Dwarf purple	Tall purple Dwarf purple (9)	Dwarf white Dwarf purple (3*b*)	Tall white Dwarf purple (9)	Dwarf purple Dwarf purple (3*b*)

II. THE RESTRICTION OF MENDEL'S SECOND LAW—LINKAGE

Mendel worked with relatively few characters in edible peas—seven pairs in all. All of these pairs assorted independently, so far as the evidence goes. Later observers have studied other characters of edible peas. White, who has in recent years worked out the inheritance of many of these characters, states that up to 1917 about thirty-two characters had been experimentally studied. He finds that certain characters do not show independent assortment but exhibit a new phenomenon known as linkage.

The discovery of linkage was made in 1906 by Bateson and Punnett in the sweet pea. Since then, many cases of linkage have been found. In animals, the most numerous linkage relations are those in the vinegar fly (*Drosophila melanogaster*). In plants there are many scattered cases of linkage (maize, snapdragon, peas, oenothera), but as yet not very many cases in any one plant are on record. A few specific examples will suffice to illustrate linkage relations.

The two mutant characters of the vinegar fly, yellow wings and white eyes, are linked as shown by the following cross. If a female with yellow wings and white eyes is mated to a wild-type male with gray wings and red eyes, all the daughters have gray wings and red eyes and all the sons yellow wings and white eyes. If these are inbred, 98.5 per cent of the offspring are like the two grandparents, that is, they have either yellow wings and white eyes, or gray wings and red eyes. In other words, the Mendelian factors that went into the cross have not freely assorted, but those that went in together have come out together in 98.5 per cent of the offspring. Thus yellow wings and white eyes are linked, and gray wings and red eyes are linked. As stated, the linkage holds here for 98.5 per cent of the offspring, but in the remaining 1.5 per cent there is an interchange, and yellow-winged flies with red eyes, and gray-winged flies with red eyes occur. The latter phenomenon, known as crossing-over, will be discussed later.

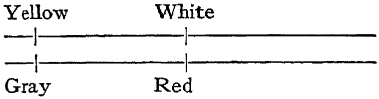
There is another mutant race in *Drosophila* with vestigial wings. Vestigial is a recessive character, and if bred to a wild-type fly (with long wings) it gives in the second generation (F_2) three longs to one vestigial. Another race of these flies has black body color. This also is a recessive character, and if bred to normal flies gives, in F_2, three normals to one black. If a vestigial fly with normal body color is mated to a black fly with long wings, the offspring, F_1, have long wings and normal body color. If the F_1 females are bred to a double recessive black vestigial male, then 83 per cent of the offspring belong to the two grandparental types, i.e., they are either vestigial with normal body color or black with long wings. Linkage is here shown by 83 per cent of the F_2 offspring. The remaining 17 per cent are cross-overs.

More than 300 mutant characters of *Drosophila melanogaster* have been studied. They fall into four linkage groups; that is, each character pair shows linkage with one group, and gives free assortment with the members of the three other groups. There are four pairs of chromosomes in each cell of *Drosophila melanogaster*. If the representative units in the germ material are carried by these four chromosomes, the linkage can be accounted for, provided the chromosomes remain to a certain degree intact through successive cell generations. Free assortment can also be explained if the units that assort are located in different chromosome pairs.

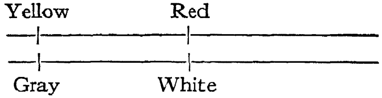
This interpretation of linkage would in itself furnish a strong argument in favor of the view that the phenomenon of linkage is due to genes being carried in the chromosomes. There is other evidence, also, indicating that the hereditary units are borne by the chromosomes.

III. THE BREAKING OF THE LINKAGE, OR CROSSING-OVER

Linkage means that when certain characters go into a cross together they tend to remain together in later generations. It has been stated above that the linked characters do not always hold together, but sometimes an exchange takes place between those that go in from one parent and those that go in from the other parent. This interchange is called crossing-over. For instance, yellow wings and white eyes and gray wings and red eyes that went in together in the illustration given above come out together in 98.5 per cent of the offspring, but in 1.5 per cent of the individuals of this generation there are two other kinds of flies. Half of these are yellow with red eyes, and half gray with white eyes. It may be said, therefore, that the genes for red and for white eyes have interchanged places. If we represent the combination that went in by lines with points indicating a position for the genes, then the original linkage relation may be expressed as follows:



If white and red are interchanged, the new relation may be expressed as follows:



Now it can be shown experimentally that the two new combinations (after the interchange has taken place) show again the same linkage relations to each other as did the former combinations. For example: If a female that has yellow wings and red eyes is crossed to a male that has gray wings and white eyes, the daughters will have gray wings and red eyes, and the sons yellow wings and red eyes. If these are inbred, 98.5 per cent of the sons are like the two grandparental flies (yellow red or gray white), but 1.5 per cent are cross-overs (yellow white or gray red). This result is important, since it shows that the linkage relation is independent of the characters that form the combination. In other words, the linkage is the same for yellow and white as it is for yellow and red. This relation holds for all the pairs of linked genes that have been tested. As will be shown later, these results find a rational explanation on the basis of position of the pairs of genes in any particular cross.

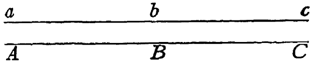
The linkage between two genes is expressed by the percentage of cases in which they are found to remain together, and the converse case of crossing-over is expressed by the percentage of cases when they have not remained together.

Thus yellow and white give 98.5 per cent of linkage and 1.5 per cent of crossing-over. It may appear, therefore, that a situation might arise in which the linkage is 50 per cent and the crossing-over 50 per cent. In such a case the result would be exactly the same as though free assortment took place. It so happens, however, that in *D. melanogaster* this situation has not arisen, although there is an approach to it in extreme cases. The explanation of the results in *D. melanogaster* is found in another phenomenon known as double crossing-over.

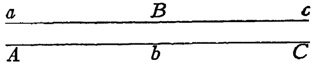
IV. DOUBLE CROSSING-OVER

The genetic evidence shows that more than one interchange may take place between the members of homologous linkage series. This fact has important consequences, for it accounts for certain relations between the genes of a series that would otherwise be inexplicable. The genetic evidence relating to the process of double crossing-over becomes intelligible if the genes lie in a single line in each linkage group.

A diagram will show how double crossing-over introduces a disturbing element into the results when single cross-overs are taken as the basis for numerical computations that involve the relations of the genes to each other. If we represent the three members of a linkage series by *a*, *b*, *c*, and their allelomorphs in the homologous series by *A*, *B*, *C*, thus,



then if one cross-over takes place between *ab–AB* and another between *bc–BC*, the result will be



If only the cross-overs between *a* and *c* (and their allelomorphs *A* and *C*) are being recorded, it is obvious that, as a result of a double cross-over, two single cross-overs will not be observed, because the end result of the double crossing-over will give *ac* and *AC*, which would be counted in with the two non-cross-over classes. Hence the number of non-cross-overs, as recorded, would be too large, with the result that the cross-over percentages will be too small as a record of all crossing-over.

How double crossing-over affects the results has been demonstrated by making the experiment in such a way that the presence of a third pair of allelomorphs, *bB*, lying between *aA* and *cC*, checks up what takes place in that region. When this is done, the double crossing-over is recorded, because two new classes appear, *aBc* and *AbC*. If the number of times that this occurs is recorded, and two single cross-overs are allowed for each double, and if these two are added to the cross-over classes, we get an accurate estimate of the number of crossings-over that occur in a given mating. This gives a higher percentage of crossing-over between two such points as *a* and *c* than is observed when there is no check on what is taking place in the region between *a* and *c*. It has been found that whenever the cross-over value of two points, such as *a* and *c*, is large, the number of double (or triple, etc.) cross-overs is correspondingly large; while if the cross-over value of two linked genes, *m* and *n*, is small, double crossing-over is small. Below a certain percentage—that is not fixed, however—the chance of double crossing-over becomes so small that it is practically negligible.

Therefore, in order to get accurate cross-over values between consecutive members of a series of linked genes, it is necessary to study regions so near together that no double crossing-over ever occurs, or else, having once determined the expectation for double crossing-over for any series, to correct the larger cross-over value by the expected amount for double crossing-over. Whenever possible, however, the former method is preferable.

The practical application of these considerations is as follows. Since the number of double cross-overs increases as the percentage of crossing-over is greater, it may happen that the observed cross-overs between two loci never go beyond 50 per cent, because the double crossing-over keeps the percentage below that number. This is actually realized in the case of *D. melanogaster.* Other cases may be expected to arise, however, in which, if the linkage series is longer or if double crossing-over is less frequent, a higher percentage than 50 per cent may be found. In such a situation the genes would show negative linkage, that is, the genes that go in together would come out more often not together. In this respect the relations may be said to pass on the other side of free assortment, which, as stated, gives 50 per cent recombination. Of course, if such a situation should arise, it would be very simple to demonstrate that the genes in question are really members of the same series, by studying their relation to other members of the series lying between them to which they will be linked. Thus, if *a* and *x* give 50 per cent or more than 50 per cent of crossing-over, but *a* is linked to *m*, and *m* is linked to *x*, it follows that *a* and *x* are members of the same series. In constructing a linear series of genes for each linkage group, this question is important, and will be further considered later.

In studying crossing-over when three points (three pairs of allelomorphs) are involved, the double cross-over class furnishes the clue as to the order of the genes. An actual illustration may show how this works out. If a fly that is sepia bithorax is crossed to a fly that is normal for these characters but has the dominant character hairless, the offspring will be heterozygous in three character pairs. If one of the F_1 females is back-crossed to a male that has the three recessive characters, namely, sepia bithorax normal hairs, the following classes of offspring are obtained; the numbers in each class being those obtained in an actual experiment:

se bx	H	se H	bx	se bx H	normal	se	bx H
216	248	106	138	32	46	17	16

The last two classes, which are the smallest, must represent the double cross-over classes, since double crossing-over is less frequent than single. Now in double crossing-over the pair of genes that has interchanged with respect to the other two is the middle pair. In the present instance it is bithorax and its normal allelomorph that are interchanged. The sequence of the genes is, therefore, sepia bithorax hairless. This conclusion can be checked up by calculating the cross-over values for the loci taken in pairs, in which case it will be found that sepia hairless gives the highest values, hence it is the longest distance involved as it should be if the order is the one stated.

V. CROSSING-OVER AND INTERFERENCE

It has been found that when crossing-over takes place in any region, the neighboring regions are protected, so to speak, from a second crossing-over. Thus, if crossing-over takes place between *a* and *b*, then to the left of *a* and to the right of *b* a second crossing-over is not as likely to occur as it is at a more distant region. In fact, immediately on each side of the cross-over, no crossing-over at all takes place. A little farther away there is a chance that crossing-over may occur, and the farther away to the left or right a region lies, the greater the chance there is of a second crossing-over. This relation holds until a certain "distance" is attained, when the first crossing-over no longer affects the chance that a second may appear. This may be regarded as a neutral point. As just stated, in terms of probability, the real meaning of the phenomenon is not at first apparent, but as soon as the situation is examined by means of a series of points that cover the entire length of the linkage series, a possible mechanical explanation becomes evident. Such a study reveals the fact that when crossing-over occurs at any level, there is an interchange between whole series or blocks of genes. Thus, when a series *abcdefgh* (and its allelomorphic series *ABCDEFGH*) breaks between *c* and *d* (and *C* and *D*), the outcome is the formation of the two series *ABCdefgh* and *abcDEFGH*. Similarly, when two cross-overs occur, let us say, between *C* and *D*, and *f* and *g*, the outcome is found to be *ABCdefGH* and *abcDEFgh*.

In any given experiment there are usually more non-cross-overs than cross-overs, which means that in many cases no interchange takes place. For the sex chromosome of *Drosophila* 43.5 per cent cases of non-crossing over are found. In about 43 per cent of cases a single cross-over occurs; it may occur at any point in the series, and when it does it involves interchange of equal portions of the two series. In only 13 per cent do two cross-overs occur, and triple cross-overs only in 0.5 per cent. It is also found from a study of double crossing-over that there is an average length of pieces that cross over. The average length of a piece expresses the likelihood of a second cross-over occurring at a certain distance from the first one, or, expressed in another way, if the average length falls within the series, a second cross-over is likely to occur at that point; but if the point falls outside the series, then only a single cross-over occurs. Similar predictions may be made for lengths, shorter or longer than the average. When the relation of crossing-over to the chromosomes is considered, it will be pointed out how this relation can be given a mechanical interpretation.

VI. THE CHROMOSOMES IN RELATION TO THE MECHANISM OF MENDELIAN HEREDITY

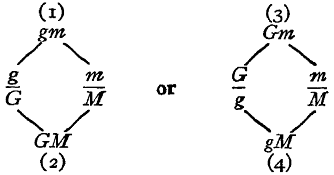
There is a great deal of evidence indicating that the chromosomes are the bearers of the mutant genes and of the normal allelomorphs of these genes, and hence are the bearers of the factors that students of Mendelian inheritance follow. It is not necessary to consider this evidence, although incidentally some of it will be referred to later when special types of inheritance have been demonstrated to be connected with the presence or absence of whole chromosomes. It will suffice to point out here that the behavior of the chromosomes at the time of maturation of the gametes supplies a mechanism that gives a consistent explanation of Mendel's two laws, and one that can be brought into relation also with the phenomena of linkage, and even with crossing-over.

If we suppose a given mutant factor to be carried by a particular chromosome, and its normal allelomorph to be carried by the homologous chromosome, then at reduction, when these two chromosomes separate, the two factors will also separate. Consequently, the gametes with the haploid number of chromosomes will contain one or the other member of the pair of allelomorphs. Such a result fulfils all the requirements of Mendel's assumption of segregation. When a spermatozoön unites with an egg, the two genes are brought together again in the zygote, which is a hybrid, if the two are different. The same process repeats itself in every generation.

In the male there are formed four sperm cells, as a rule, from each spermatocyte. Since the reduction in the number of the chromosomes takes place at only one of the two divisions, half of the sperm will contain one member of each pair of chromosomes, and the other half of the sperm will contain the other member of the same pair. Thus, two spermatozoa contain each one of the chromosomes, and the other two spermatozoa contain each one of the other chromosomes. If the chromosomes contain the pairs of genes, this result fulfils Mendel's requirement for the male.

In the female the results are essentially the same, but since only one of the four cells produced at maturation becomes the egg cell, and the others are lost, a further statement is necessary. If the reduction occurs at the first division, one chromosome of each pair goes into the first polar body, the other remaining in the egg. The former is lost, but the latter, by an equational division gives rise to two like chromosomes. Of these two, one is lost in the second polar body, and one remains in the egg. Since either member of a pair of chromosomes may go out of, or remain in, the egg, it follows that half the eggs will contain one member of a given pair, and the other half of the eggs will contain the other member. Stated more generally, half the eggs contain one chromosome of each pair, and half the other member. This result fulfils Mendel's requirements for the egg.

Whenever a mutant gene and its allelomorph are present in one pair of chromosomes, and another mutant gene and its allelomorph are present in another pair of chromosomes, the behavior of these two pairs of chromosomes at reduction would explain Mendel's second law of free assortment, *provided* the two pairs of chromosomes behave independently of each other. An illustration will make this clear. If a gene, *g*, and its allelomorph, *G*, are carried in a pair of chromosomes, and another gene, *m*, and its allelomorph, *M*, in another pair, the two pairs may be represented on the reduction spindle in either of the two following ways:



Four and only four possible kinds of gametes result from the separation, namely, (1) *gm*, (2) *GM*, (3) *Gm*, and (4) *gM*. This is Mendel's second assumption for free assortment.

That the pairs of chromosomes separate at random during the reduction division has always seemed probable. The alternative would be that the entire maternal group of chromosomes goes to one pole of the maturation spindle, and the entire paternal group to the other. The demonstration that the pairs freely assort was first clearly shown by Carothers (1917). This evidence shows that the behavior of the chromosomes at reduction is such that if the genes are carried by them, each pair will be distributed according to Mendel's first law, and if two or more pairs of genes are carried by different pairs of chromosomes, they will be freely assorted, according to Mendel's second law.

Linkage of the genes carried by a chromosome is expected if the chromosomes remain intact through successive cell generations. It has been difficult to demonstrate that the chromosomes do remain intact during the resting stages of the nucleus; while this assumption would offer the simplest interpretation of the reappearance of the same number of chromosomes, having the same shapes and sizes, at each mitosis, yet this might be due to other relations than that of continuity. In fact, the spinning-out of lateral branches from the chromosomes as the nucleus re-forms after each division (so often described by cytologists), and the apparent network that results is difficult to harmonize with the view that no interchange of materials takes place at such times. The fact, pointed out by Boveri, that in *Ascaris* sister-cells show mirror pictures in the arrangement of their chromosome loops, appears to indicate that the main centers or lines of the chromosomes at least do remain in position while in the resting nucleus, but does not prove conclusively that no interchange takes place. Again, such figures as those for *Phrynotettix* given by Wenrich seem to indicate that for a long time each chromosome has a distinct nuclear area to itself, but since these vesicles cannot be followed from one cell generation to the next in the same cells without any loss of continuity of observation, the possibility remains open that there may be a time when the areas become more or less continuous. The *X* chromosome in the male of certain insects forms a separate sac by itself during the growth period of the germ cells. It appears, therefore, to be isolated from its fellows, but since throughout the earlier divisions the *X* chromosome has not been so separated between mitoses, it is impossible to appeal to this particular case as demonstrative.

Thus, because cytologists have not yet been able to prove beyond all question the "individuality" of the chromosomes, the geneticist is left without the support that his evidence calls for. In fact, genetics has shown that if the chromosomes are the bearers of the genes, there must at some time be an interchange between members of the same pair. Janssens (1909) has attempted to show how such an interchange might take place, but his interpretation has been questioned by other cytologists. It is a misfortune for genetics that at present students of the cell are not agreed as to any one period or method by which interchange between the members of the pairs of chromosomes might occur.

For the present, at least, we shall have to reverse the situation, and argue that since there is excellent evidence that the chromosomes carry the genes, the chromosomes must remain intact, except in so far as crossing-over takes place between homologous chromosomes.

The agreement between the number of linkage groups and the number of chromosome pairs is a relation of the utmost importance for the chromosome interpretation. It is fortunate that in one form at least this relation is so clearly established that no one can seriously doubt its significance. That the 350 known genes of *D. melanogaster* fall into four linkage groups, and that there are only four pairs of chromosomes (Fig. 1), can scarcely be regarded as a coincidence, especially when other evidence has shown, experimentally, that one of these groups is the *X* chromosome and another group is the small IV chromosome.

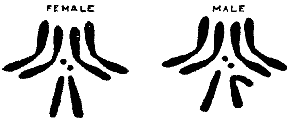


FIG. 1.—Female and male groups (diploid) of chromosomes of *Drosophila melanogaster*.

Furthermore, the same relation has now been established in other species of *Drosophila*. For instance, *D. virilis* has six pairs of chromosomes and the same number of linkage groups; *D. willistoni* has three large and possibly one small chromosome (IV) and three known linkage groups; *D. simulans* has three large and one small chromosome (IV) and three linkage groups. The last species alone can be crossed to *D. melanogaster*, and by this means Sturtevant has shown that many of the mutant types in the two are identical and in the same linkage groups. In a few other cases also there is an approach to agreement. In the edible pea there are seven chromosomes, and Pellew and Sverdrup report three linkage groups; in the sweet pea, with seven chromosomes, Punnett has found seven character pairs that assort freely and possibly one other independent character that may, as he points out, be only loosely linked to one of the other groups. In maize and in the snapdragon the number of independent character pairs approaches at present the number of the chromosomes.

VII. THE MECHANISM OF CROSSING-OVER

In order to determine the mechanism of crossing-over it was very important to discover, if possible, the time at which it occurs. If it is granted that the chromosomes carry the genes, and that the two homologous linkage series are carried by homologous chromosome pairs, then it seems probable that such an interchange could most easily take place at the time when the members of each pair conjugate with each other. An experiment planned with *Drosophila* to get evidence on this point was carried out by Plough (1917) in the following way. It was known that when the female emerges from the pupa, a certain number of eggs—about 150—have passed through the maturation stages. There is rather a sharp distinction between these eggs that lie in the posterior ends of the ovarian tubules and those in front of them. It was known, also, that crossing-over in the second chromosomes is influenced by temperature. If, therefore, a female has been reared from egg to adult at a given temperature, the crossing-over that has been completed in her most advanced eggs at the time of her emergence must correspond to that characteristic for the temperature. If, now, she is changed to another temperature, the remaining eggs will pass through the maturation stages under the influence of the new temperature, and should show a different percentage of crossing-over from the first set, if crossing-over occurs in the maturation stages. The results showed, in fact, that a change in the percentage of genetic crossing-over took place at this time, and it is probable that chromosomal crossing-over takes place at the same time. The results are so important for the question under consideration that further details may not be out of place.

A female was made up in which one of the second chromosomes carried the recessive genes for black, purple, and curved, and in which the other second chromosome carried the normal (wild type) allelomorphs of these genes. She had been reared at a high temperature (31.5°) that gives a high cross-over value. She was crossed to a male homozygous for the three recessive genes in question, and transferred to room temperature (22°C.). Her first output, extending over ten days, gave a high cross-over value which dropped during the next ten days. This result shows that a change in temperature does not affect those eggs that have passed through their maturation stages, and that those eggs that have not reached this stage are affected.

In another experiment, the same kind of heterozygous female had been reared to maturity at room temperature. She was then mated to a triple recessive male (as above), and placed at a high temperature (31.5° C.). The first batch of eggs gave cross-over percentages that are characteristic for room temperature. About the eighth day the effects of the higher temperature began to show, and lasted for ten days longer.

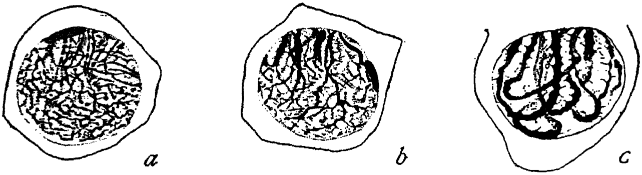


FIG. 2.—Three stages in the conjugation of the chromosomes of *Tomopteris* (after Schreiner).

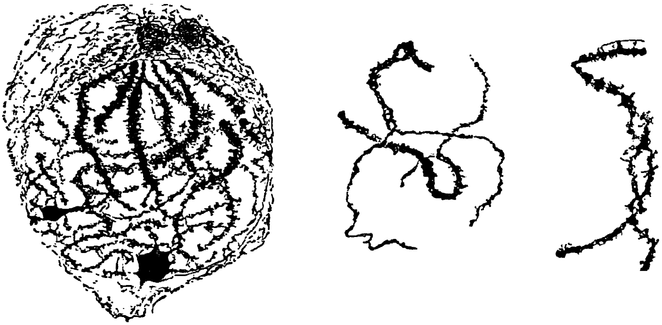


FIG. 3.—Conjugation of the chromosomes of *Batracoceps* (after Janssens)

These results prove not only that eggs that have passed through the maturation stage do not show the effect of a change to another temperature, but also that the early stages of eggs, i.e., those before the maturation period, are not affected by temperature changes. It follows that at about the period in the growth of the egg when conjugation between homologous chromosomes occurs crossing-over may be affected by temperature conditions prevailing at that time.

These conclusions, while giving important information as to the time of crossing-over, do not furnish evidence as to the particular stage of maturation at which the process takes place. The cytological evidence shows a series of processes going on at this time. In many animals the chromosomes become greatly elongated at the beginning of the period, and are correspondingly thin. In this condition they conjugate (Fig. 2*a*, *b*). If the chromosomes are bent rods or loops, the free ends of the loops first come together side by side, and the union continues until the two loops have united into a single one. Some of the figures of these stages show that the chromosomes overlap when the threads are about to come together (Fig. 3), and several accounts describe the thin threads as actually twisted about each other, forming a double spiral. The evidence for this spiral twisting is, however, by no means satisfactory. When the thin threads have fused into a single thread, all evidence of a spiral structure is lost (Fig. 2*c*), but if the union does not mean a real fusion, but only a side to side apposition of the two threads, it does not seem improbable that the two threads may at times remain actually twisted about each other.

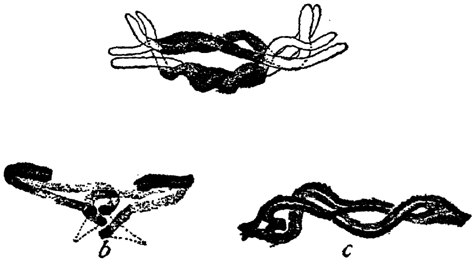


FIG. 4.—Contrated phase of the chromosomes of *Batracoceps* when ready for the first maturation spindle (after Janssens).

Somewhat later, the double chromosomes are found in the form of two shorter threads twisted about each other (Fig. 4), but it is not known whether

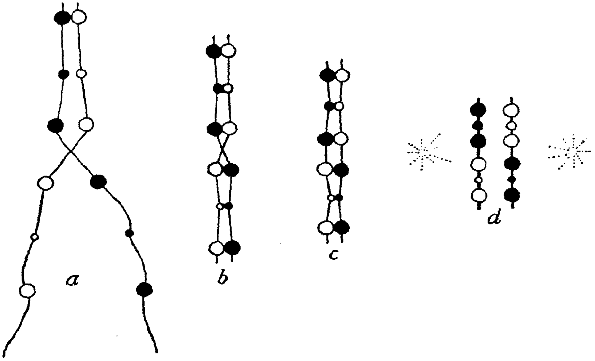


FIG. 5.—Diagram to illustrate single crossing-over in the thin-thread stage

this secondary twisting represents an earlier twisting of the thin thread, or is a new phenomenon connected with the shortening of the threads.

If the genes lie in linear order it would seem a priori more probable that the crossing-over has occurred at the thin-thread stage (Fig. 5), when the thread has its greatest extension. At least fewer mechanical difficulties are met with on this assumption. Moreover, the phenomenon of the extension of the threads occurs only at this period in the whole life-cycle of the cell, and at none of the other division stages, so far as known. This creates a prejudice in favor of looking to this stage as the more probable stage of crossing-over, rather than to the later ones, when the visibly twisted threads have greatly condensed, but at present this is only a surmise, and not a fact of observation.

There is another occurrence that has been often described for the synezesis stage, namely, the splitting lengthwise of the conjugating threads, each into two strands. When this splitting occurs is a matter of dispute at present among cytologists. According to one view, it has taken place during the final stages of the preceding division, as the double chromosomes are moving toward the poles. According to another view, the splitting of the threads does not occur until the time of conjugation of the threads, or even after they have conjugated (Fig. 6). The splitting is generally interpreted as the forerunner of the split for a later division, i.e., the formation of two daughter-threads out of a single one. If it occurs at the earlier stage, and if the double threads pass into the resting nucleus in this condition, it might appear, at first sight, that this would give an opportunity for interchange between the strands, but such a result would not in any way represent crossing-over, which takes place, not between strands of the same chromosome (although this, too, may happen sometimes, as we know), but between strands of different chromosomes (of the same pair). The situation is involved by the further consideration as to whether crossing-over takes place only between two of the four strands of chromosomes, or between whole chromosomes, whether split or entire. From the genetic point of view the outcome would be in most cases exactly the same, but the question cannot be ignored, because the most familiar evidence of crossing-over that cytology has to offer (Janssens, 1909) is based on the assumption that the strands rather than entire chromosomes interchange.

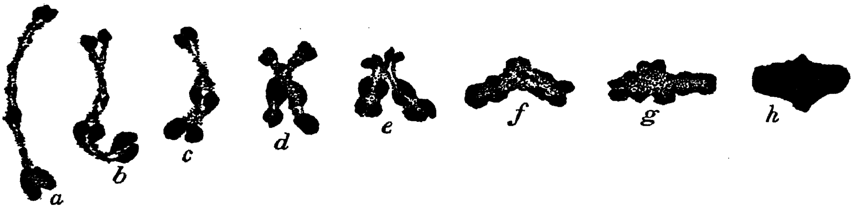


Fig. 6.—Conjugated chromosomes of *Phrynotettix*. Each thread (chromosome) is split lengthwise into two strands (after Wenrich).

Janssens described the condition of the chromosomes of the salamander *Batracoceps* during the maturation period of the male germ cells. After the single threads have united, beginning at the ends of the V's (Fig. 3), the double chromosomes pass on to the spindle. At about this time the double chromosome is found to be split into four strands throughout its length. Presumably, one of the splits corresponds to the plane of conjugation (the reductional split), and the other to a split in each member of the pair (the equational split) (Fig. 7). During the two cell divisions that follow, the separation of the strands occurs along these lines of splitting.

In addition to these familiar conditions, Janssens described certain configurations found in the contraction phases (Fig. 7*e*) that may be interpreted as the result of crossing-over of two of the strands at some early stage during the conjugation process, but there are two possible interpretations to be placed on figures of this sort that are illustrated in the diagrams of Figures 7 and 8. In Figures 7*a*, *b*, two chromosomes (black and white), each split lengthwise, are represented as overlapping each other. If, where the threads cross, only the two strands that touch each other break, and the broken end of one strand unites with the broken end of the strand of the other chromosome (Fig. 7*b*, *c*,), the process of interchange is accomplished. The threads now come to lie side by side throughout their length (Fig. 7*d*). In this condition the chromosome is supposed to pass into the spindle. As a result of the earlier crossing-over the strand will appear, when it later opens out, as shown in Figure 7*e*. Two strands are now seen crossing-over each other near the level at which the earlier crossing-over took place, but the crossed strands are the non-cross-over strands, while the two strands that have interchanged (the real crossing-over) do not now overlap each other.

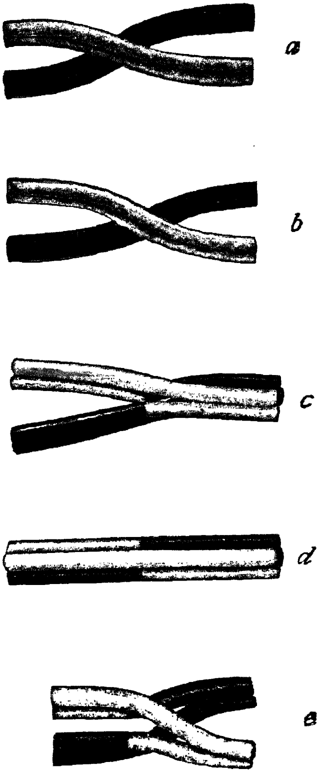


FIG. 7.—Diagram to illustrate single crossing-over involving only one strand in each chromosome (when in the four-strand stage).

But another interpretation of the crossed strands seen in the contracted phase is possible, as suggested by Robertson. This is illustrated in the following diagram (Fig. 8*a*). The four strands are represented in *a* as coming together without crossing-over in any sense (Fig. 8*b*). When they open out on the spindle, preparatory to separation (Fig. 8*c*), the opening-out is represented as taking place in different planes—in one of them between unlike strands, and in the other between like strands at *y*. As a result, two strands appear as though crossing-over, but this crossing of two strands cannot be interpreted as related to an earlier, or real crossing-over (interchange) between two strands of the two members of the pair, because no such crossing is here supposed to have taken place.

The first interpretation (Fig. 7) gives an explanation of the opening-out of the chromosome (tetrad) in different planes in different regions along its length; for, if the opening-out represents a reductional separation, then, as a consequence of the earlier overlapping of two strands at one or more levels, such a configuration of the strands is expected to follow. On the second interpretation (Fig. 8), one must suppose that the opening-out is reductional at one level and equational at another level. The future must decide between these two possible interpretations.

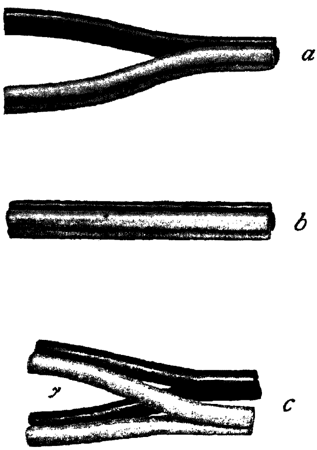


FIG. 8.—Diagram to illustrate the opening-out of the four strands in two planes giving the appearance of crossed threads.

From the genetic point of view, it makes very little difference whether crossing-over involves whole chromosomes, or only two of the four strands, because in both cases only one of the four strands is left in the gametes. In the male, where all four strands are preserved—one going to each gamete—there will be four cross-over gametes from each spermatocyte if whole chromosomes have interchanged, while if only two strands have interchanged, there will be two cross-over and two non-cross-over gametes. In the former case it might appear that there was twice as much crossing-over as in the latter; but since crossing-over is measured by the number of cross-over individuals, without regard to whether it took place in two strands or in two whole chromosomes, the outcome is the same.

Up to this point only single crossing-over in the chromosomes has been examined. Double crossing-over is only an extension of the same process. If double crossing-over is represented by a double overlap (or a twist) between members of a pair of chromosomes, as shown in Figure 9, there should be a limiting value between consecutive levels at which crossing-over occurs, because of the semi-rigidity of the material. Therefore, if crossing-over is due to overlapping or twisting of the chromosomes about or around each other, the region on each side of a cross-over is protected from a second one. As this distance increases, the protection due to the stiffness of the chromosomes is gradually lessened, and then finally lost. This furnishes a picture of what the genetic facts reveal in respect to multiple crossing-over, and, from a theoretical point of view, at least, lends some probability to the view that the actual process is the result of looping or bending of the chromosomes.

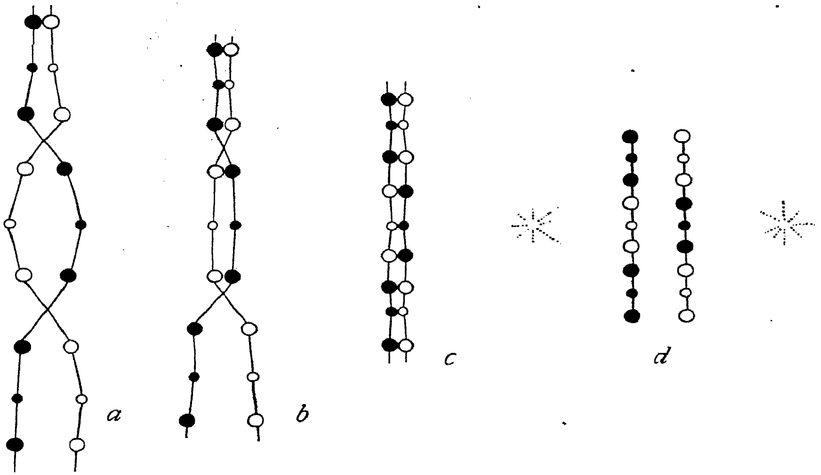


FIG. 9.—Diagram to illustrate double crossing-over in the thin-thread stage. The nodules along the strand are not intended to represent genes.

VIII. OTHER THEORIES OF CROSSING-OVER

It has been pointed out that Plough's results indicate that crossing-over takes place at about the time of maturation, but before this evidence was obtained, Bateson and Punnett had suggested that linkage and crossing-over (or coupling and repulsion, as these two relations were then called) takes place at some earlier stage in the germ track by a sort of dichotomous division—an imaginary process that was not necessarily one involving cell divisions. Aside from the fact that this view is entirely divorced from any known process taking place in cell division, there is the additional fact that the kind of ratios on which the hypothesis rests have not been ones that a fuller knowledge of the process has brought to light.

Goldschmidt suggested at one time a hypothesis based on the erroneous assumption that interchange takes place only between those genes that show crossing-over, but it was known even at that time that the essential nature of the process is such that whole pieces or blocks of chromosomes are interchanged.

Castle, also, has given an interpretation different from that based on the linear order of the genes, but his view rested on a misunderstanding of the data, as has been pointed out by Morgan, Sturtevant and Bridges, and Castle has himself partially withdrawn his interpretation.

Seiler has recently observed a process in the maturation of the germ cells of certain moths that would explain crossing-over to a limited degree. For instance, in the male moth of the *Solenobia pineti*, there are two chromosomes, presumably sex chromosomes since only one of these occurs in the female. These chromosomes at certain stages in maturation of the sperm cells are detached and later reattached to the members of a certain pair of chromosomes (autosomes or sex chromosomes, according to definition). Now if these free chromosomes sometimes change their attachment, the result will be the same as when crossing-over occurs between chromosomes; only here if the breaking apart is at a fixed point the result will only give a sort of crossing-over for that level.

The scheme of chromosomes crossing-over here followed rests on the common observation that side-to-side union of the chromosomes occurs. It will not apply to cases, if there are such, in which the reduction in the number of chromosomes is brought about only through an end-to-end union (telosynapsis). There are a few accounts of reduction taking place in this way, especially in plants. Now if it could be shown that genetic crossing-over takes place when end-to-end union takes place, it would follow that our scheme, that depends on lateral conjugation, could no longer stand. On the other hand, there are indications in a few cases that the observed end-to-end union of the chromosome sometimes represents the final phase only of a conjugation process that is essentially a side-to-side union. Therefore, until these questions are put on a more satisfactory basis by cytologists, the argument against crossing-over from end-to-end union need not be considered as a serious objection to the twisting hypothesis.

When it was found that crossing-over occurs in the female of *Drosophila*, but not in the male, and that in the silkworm moth the crossing occurs in the male (Tanaka) and not in the female, the question arose as to whether crossing-over bears any relation to the fact that in the former the female is homozygous for the X chromosomes, and in the latter the male is homozygous for the Z chromosomes. It is not, of course, obvious why such a relation should affect crossing-over, but if it did occur between the X and the Y in the XY male, the sex mechanism would be disturbed. If, as is said, crossing-over occurs in mice and in fish, both in the male and in the female, the sex mechanism requires, at least, that crossing over does not take place between the X and the Y. The Y, for example, may be absent.

In hermaphrodite plants there is evidence of crossing-over both in the egg and pollen cells, and the percentage of cross-overs may be different in the two kinds of germ cells (Altenburg, Gowen). What special conditions determine whether crossing-over occurs in one or in both sexes is not known, but there is now abundant evidence in *Drosophila* to show that crossing-over is a very variable phenomenon, and may be influenced by external as well as internal genetic factors. For example, it has been shown that crossing-over changes with the age of the female, and that it is also affected by temperature. Moreover, there are known to be genetic factors that interfere with the percentage of crossing-over, and these factors may be carried either in the pair of chromosomes that are themselves affected (Sturtevant, Muller, Bridges, Gowen, Ward) or in other chromosomes (Sturtevant and Gowen). Moreover, there are genetic factors that influence crossing-over in particular regions of certain chromosomes —factors carried by the regions themselves. There are also other kinds of influences, such as deficiencies and duplications, that interfere with crossing-over. It is quite clear, therefore, that no emphasis is to be put on the reversal of crossing-over relations in the *XX–XY* type, as represented by *Drosophila,* and in the *WZ–ZZ* type, as represented by the silkworm.

IX. LOCALIZATION OF THE GENETIC FACTORS

It has been found that the cross-over values of a linkage group, if interpreted as distance along a line, give an entirely consistent scheme that is both a description of the facts, and allows one to predict the relation of any new member of the series to all the other members of the series, if its cross-over values with respect to any two of them be first determined. A simple example will serve to illustrate this statement. Yellow wings and white eyes of *Drosophila* have a cross-over value of 1.5 per cent.

If a new character, bifid wings, for instance, is found to be linked to white, giving a cross-over value of 5.8, its relation to yellow is expected to be the sum of 1.5 and 5.8 if it lies beyond white (as in the diagram below), or to be the difference between 5.8 and 1.5 if it lies on the opposite side of white. Experience shows that in such cases one or the other of these relations holds. In the present case it is the sum of the two. The following diagram shows these relations in a graphic form.

Yellow
1·5 < White > 7·3
5·8 < Bifid

Suppose now that a fourth character, cross-veinless, is added to the linkage group, and suppose that it gives a value of 6.4 with bifid and a cross-over value of 12.2 with white. It must lie beyond bifid. Hence, one can predict that when crossed to yellow it will give the sum of the three cross-over values (1.5+5.8+6.4), and this turns out always to be the case. The relation of these four characters is represented in the following scheme:

Yellow
1·5 < White
5·8 < Bifid > 13·7
6·4 < Cross-veinless

These relations are geometrically those of points on a line, and cannot be expressed in any other simple way. We are justified, therefore, in representing the linked series as such points.

The example just given may, however, give an exaggerated idea of the accuracy of these relations. In such a simple case as that of yellow-white bifid, the yellow bifid cross-over is necessarily the sum of the yellow-white and the white bifid cross-over, when all the data are taken from the same experiment; but if the data for white bifid are taken from one experiment, and those for the yellow white from another, the sum of the two may not be exactly the yellow bifid cross-over value, but only approximately the same. This is owing to a certain amount of variation of crossing-over under different conditions. This variability may happen in particular instances to be so great that the sequence of the three, as given, might not be apparent. Therefore, in order to get accurate data for the order of the genes, it is necessary to make use of only those experiments where all the conditions both external and internal are as nearly uniform as possible. It is from such data that the order of the genes is to be determined.

Since the results hold only when these conditions are present and may not always be the same, the most useful cross-over values for practical purposes will be those most frequently met with rather than those from a particular experiment. They will be represented most nearly by the average results of many experiments in which they are known not to depart very far from what ordinarily occurs. For example, cases where lethal factors are known to be present are to be excluded, also cases where environmental conditions are extremely poor, and proportionally too many of the weaker types die. Especially to be excluded are those cases where it can be shown that internal cross-over modifiers are present.

It has been shown that the actual length of the section between the genes is only one of the factors determining the amount of crossing-over between the loci, and, consequently the map distance. Both environmental and genetic factors are known to influence the frequency of crossing-over within a given length. Therefore, the length of a section of the chromosome represented by a unit distance (1 per cent) may be different in different regions of the chromosome. A parallel to the maps is found in a railroad time table, where the number of minutes between the stations is given. From such a time table one can judge accurately the sequence of the stations and roughly the actual number of miles between them. Knowledge of the speed of the train and of the condition of the road-bed and of the grades would make it possible to judge more accurately the number of miles between the stations from the number of minutes between the stations.

X. CYTOLOGICAL ASPECTS OF THE THEORETICAL ORDER OF THE GENES

Since the chromosomes themselves are rodlike or threads, the genetic evidence in favor of a linear order finds a situation favorable to its requirements. Nevertheless, it must be clearly understood that at the present time there are many aspects of the chromosome situation that must be worked out before we can go beyond the statement of a rather general agreement. It has been suggested that crossing-over takes place when the chromosomes are at their greatest extension, the thin-thread stage. This extension occurs only once in the whole history of the chromosome, so far as is known. Whether the linear order is maintained when the chromosome condenses, we do not know. Until this and other questions can be answered, it would not be profitable to speculate further concerning the applicability of the conclusions from the genetic evidence to the rather meager facts supplied by cytology.

XI. THE GENE

The extensive use of the term unit character, and its representative, the gene, in the germ material, led at first to an unfortunate and widespread impression that for each unit character there was supposed to be one and only one unit or element in the germ material. Weismann's theory of the germ plasm that aroused much interest, and also much adverse criticism, was still in the air, and it appeared to many biologists that the Mendelian gene, as the representative element in the germ material, responsible for the unit character, was not very different from Weismann's determinant. Hence a part of the antagonism aroused by Weismann's speculation was transferred to Mendel's theory. Although the opposition has gradually subsided owing to a better understanding of the difference between the two views, nevertheless it is important to make entirely clear that the modern theory of the gene has only a superficial resemblance to Weismann's theory of determinants. Both start from the common assumption that the germ material is not to be considered as a single unit, but as a complex of many units. Aside from this general similarity, the interpretations of the relation of the units to the characters are entirely different. Weismann held that as the embryonic development goes on, or, more specifically, as the egg segments, the units called determinants, are sorted out so that each kind finally reaches that part of the embryo where it is to produce the particular character that it represents. Weismann's entire theory of pre-formation was utilized primarily to account for embryonic development by means of the sorting out of the pre-formed elements during ontogeny. His explanation was purely formal. He made no attempt to explain how the architecture of the germ plasm in the fertilized egg could be of such a kind as to undergo the orderly disintegration process demanded by his view. It was not long before numerous facts from experimental embryology demonstrated the incorrectness of this speculation. The disrepute, into which Weismann's speculations then fell, carried over, as I have said, for a time at least, and prejudiced needlessly the Mendelian situation. The difference between Weismann's theory, as to the relation between the postulated units in the germ material and the characters of the embryo, and the present so-called Mendelian theory (although in reality a later development out of Mendelism) calls for further discussion.

If it be granted that in a given environment the individual is the end product of what is present in the egg, then it is evident that any change in the materials of the egg may be expected to give a different result. Whether the entire end result will be affected, or only some part of it, will depend on the nature of the change. Experience shows, as a matter of fact, that when an alteration takes place in the germ material that gives rise to a new stable condition, i.e., an alteration that is inherited (mutation), the individual that results may be changed visibly only in one small part or else in many parts, and the changes may be minute or mediocre or very great. When the new individual differs sufficiently from the race from which it originated to be distinguishable from the original type, it is called a mutant, and if in breeding back to the original type it is found to give a three-to-one ratio in the second generation, we explain the result by stating that the two kinds of individuals differ in only a single factor. Since it is manifestly impossible to take account of all the differences between the new and the old types, and since the differences are often more marked in one part of the body than in another, it is customary to use a conspicuous character as the symbol of the change in the germ material, and to baptize the new organism (either in Latin or in the vernacular) with the name of this particular character. It is this procedure that is responsible for the much-abused expression unit character—a term well suited to express the contrast between the old and the new type of character, but entirely misleading if it is taken to imply that the character is the sole product of a single element in the germ material.

To what extent there is in addition to the general effects of each gene a more pronounced specific effect is illustrated by those cases in which more than one change has taken place in the same locus. At present there are several cases of this sort known. Thus, there are ten different eye colors in *Drosophila*, each due to a change in the white locus. There are five different mutations in the ebony locus; four in forked; four in pink; four in lozenge; about five in cut and four or five in truncate; three in singed, etc.[1] In fact, it has been found that when a locus changes it affects primarily the same organ in the same direction. This evidence is explicit, and has a very important bearing on the question under consideration. It means that the genes are specific for certain of the end products of development, and may have effects in other parts of the body as well. It would be, however, entirely erroneous to conclude from this relation that each character is the product of a single gene. The development of any part of the body must be the result of the co-operation of many genes—how many we do not know—but since any organ or structure may be affected by a change in any one of a large number of genes, as the evidence from *Drosophila* makes plain, it is rational to suppose that most of the genes before they were changed also had an influence on the organ or character in question.

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[1] Genes of multiple allelomorphs are known in a few other forms, but in none of them except in maize has their independent origin from the wild type been established. Without this information such cases cannot always be distinguished from cases of closely linked genes.

These and other considerations, that need not be elaborated here, lead to the conclusion that germ material, transmitted by the chromosomes, is composed of a number of different elements, genes, that affect the development in various ways. The evidence shows that while each gene may have a specific effect on certain parts of the body, it may also have other effects on other parts of the body. Furthermore, each organ or character is the end result of the action of many genes. In fact, each part may be said to be the end product of the activity of all the genes—each one contributing something to it at one or at many stages of its development. This conclusion postulates a very different relation between gene and character from that assumed by Weismann.

The embryological evidence points unmistakably to the conclusion that at each cleavage of the egg the chromatin material is divided equally, and that every cell of the body receives the sum total of the genetic materials carried by the chromosomes. This evidence is in full accord with the conclusions drawn above, for the whole genetic complex is present in every part of the body at all times. Whether all the genes are functioning all the time, or only begin to function when in the course of the progress of embryonic development new structures arise, we do not know, but however this may be, it is evident that since all the genes are present, the development of every part may be affected by the presence of all or of any of them.

XII. THE ADDITION OR LOSS OF WHOLE CHROMOSOMES

One of the most recent developments of genetics is concerned with the effects produced by a change in the number of chromosomes. The results are important both because through a change in the chromosome groups there is introduced the possibility of endless permutations of the genes, involving both additions and subtractions of their total number, and because the conditions that result from such kinds of change have an important relation to the interpretation of mutation through a change in a gene.

In cases where haploid, triploid, or tetraploid individuals occur, the total number of genes is changed, but the same kinds of genes are present. Under most circumstances the resulting individuals would be expected to be identical, but even here further complications arise from two principal sources. First, the relation of the genes to the amount of protoplasm may or may not introduce differences in the developmental aspects of such situations. Second, the balance between the genes may be of such a kind that the haploid, diploid, triploid, and tetraploid do not represent identical relations among all the genes; for if the amount of all the genes is doubled at the same time it does not follow necessarily that there is produced the same proportionate effect on the end product. However, since the diploid and tetraploid types are usually not very different in appearance, it seems that most genes do give a nearly proportionate effect under these circumstances.

The most familiar case of the effects of a change in the number of the chromosomes is that of the male and female in those cases where the female is *XX* and the male *XO*. The difference of an *X* here throws the development in one or the other direction, so far as the sex differences are concerned, yet in some cases alters the individual in other respects to such a slight degree that except for the genitalia and the gonads great difficulty may be found in distinguishing the sexes. This situation may be regarded as the extreme in one direction. There are other cases as extreme in the other direction, where the difference in one chromosome may cause the male and the female to develop into such different kinds of individuals that their relationship would not be suspected were not the history known.

In the few instances among animals where one sex is haploid and the other sex is diploid (bees, ants, wasps, and rotifers) there is an excellent illustration of the development of an individual with only one set of chromosomes into an organism as complete as when both sets are present (in the bees, at least).

In the case of hermaphroditic types, where both ovaries and testes develop, there is still a different situation, for here in the presence of the entire (diploid) group of chromosomes an ovary develops at one level and a testis at another. We are justified, perhaps, in referring such cases to the same process that determines in the development that one region of the embryo produces one kind of organ and another region a different kind of organ in the presence of all the hereditary factors, but at present we know very little as to the nature of the factors involved. There is nothing self-contradictory in the view that in different animals different relations have been established by which the same or very similar end results are reached, but it by no means follows that, when one or another of these differentials has become established, the results may be changed by any sort of environmental change to which the egg or embryo is subjected. The adjustment must be supposed to be so well established in such cases that it may not be easily changed without affecting injuriously the entire product of development. The most familiar effects are those produced in hermaphrodite forms, when, through environmental changes, development of one or the other gonad may be suppressed. A case of this kind is found in the gephyrean worm *Bonellia*, where, according to Baltzer, the embryo may become a functional female unless it settles down on the proboscis of an older individual, when it becomes a male. If this result is confirmed, it shows how in such a hermaphrodite type the environment determines the kind of individual that develops, which in turn brings about the suppression of the male or female gonad respectively. It is hazardous to argue from a unique situation of this kind to other types where the regulation has been adjusted in other ways.

The old discussion as to whether all organisms are potentially both male and female is one of those sterile transcendental questions over which one need no longer waste time in discussion. What we need to ask today in any situation is what particular set of regulations determines whether an egg develops into a male or into a female. Perhaps the most significant fact in this connection is the discovery that a genetic factor may cause the change of a hermaphrodite form into a unisexual form as in *Lychnis* (Shull) and as in the strawberry (Valleau). While at present there is no genetic evidence of the change from unisexual to hermaphrodite, there is anatomical evidence that in some nematodes and barnacles and in the fish *Serranus* such a transformation has taken place.

Aside from the situation in which a sex chromosome serves as a differential for producing two kinds of individuals, there are a few instances known to us where the addition or the loss of a chromosome is compatible with the survival of the individual that possesses it or lacks it. The first case of the kind was that of *Oenothera lata*, in which fifteen instead of fourteen chromosomes occur. The presence of the extra chromosome induces certain changes in the plant that suffice to distinguish it at once from the typical *O. Lamarckiana*. The presence of an unpaired chromosome gives rise to a kind of inheritance that exactly parallels the production of males and females in the *XX–XO* type, for the unpaired chromosome goes at the reduction division to only half the gametes; hence, when such a form is crossed to the normal, two kinds of offspring are produced, one with and the other without this chromosome. The most fully worked-out case of the effects produced by the addition of one more chromosome of each kind to the diploid series is that of *Datura* described by Blakeslee, Belling, and Farnham (1920, 1921). This plant has twenty-four chromosomes (the haploid number is twelve). By the addition of one of these to the set (giving twenty-five chromosomes) a different kind of plant results. There should be twelve such mutations possible, and, as a matter of fact, Blakeslee (1922) reports twelve types, in each of which there are twenty-five chromosomes, and at the reduction period each has been shown to produce gametes with twelve and with thirteen chromosomes.

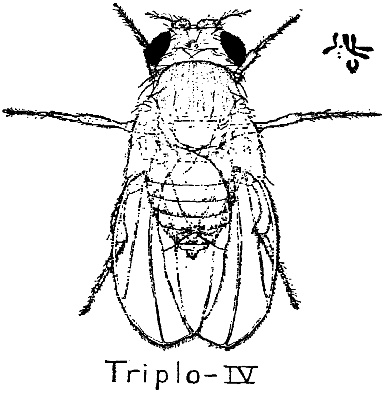

FIG. 10.—A triplo-IV fly (*D. melanogaster*). The group of chromosomes of this triplo-IV fly is drawn in the upper right-hand corner.

Individuals with three of the small chromosomes of *Drosophila* (IV) have been found (by Bridges) to occur rarely. When three are present, the fly possesses certain characters that distinguish it from the wild-type fly in a number of minute differences (Fig. 10). At the reduction division in such a triplo-IV fly, two kinds of gametes are expected, one with two, the other with one, chromosome IV (Fig. 11). If such an individual is crossed to a normal, the offspring are of two kinds, one having three IV's the other two IV chromosomes (Fig. 11). The former continues the triplo-line, the latter is a normal individual. When two triplo-IV individuals in which one of the three IV chromosomes carries a recessive factor are mated, the ratio of dominants to recessives in the offspring is not 3:1 but about 26:1.

The reverse situation is found in another type of *Drosophila,* in which one chromosome IV is absent. Here also its absence gives rise to a number of minute differences that distinguish this fly from the wild type (Fig. 12). Owing to the absence of the mate of chromosome IV, half the gametes will lack it, and half possess one IV. Consequently, if bred to wild-type stock, the offspring are of two kinds, one kind normal with two chromosome IV's, the other kind with only one IV. These haplo-IV flies also give an interesting result if crossed out to a stock that carries a recessive factor in chromosome IV. Half of the offspring will show the recessive character. These are those arising from the egg without a IV, fertilized by a sperm that carries the recessive gene. In a sense this recessive might be said to be dominant in half the offspring, but, in reality, the absence of the normal allelomorph of the recessive is responsible for the apparently exceptional behavior of a recessive character. Incidentally, this result shows that one dose of this recessive gives the same result as do two doses in the typical cases. However, the recessive character is always modified somewhat, "exaggerated," by the loss of genes of one IV chromosome.

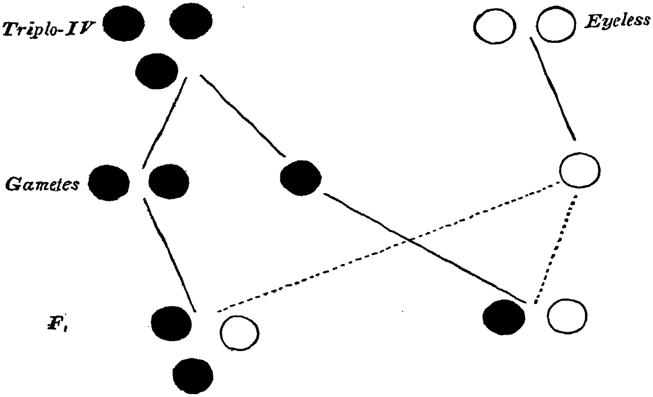

FIG. 11.—Diagram to illustrate a cross between a triplo-IV fly and a normal diploid individual. The three IV chromosomes of the triplo-IV are represented by the black circles; those of the diploid by the two open circles. In the second line the gametes of each are shown; and in the third line the two resulting flies (with respect to the IV chromosome).

The fact that it is the small IV chromosome that has given rise to the two situations just described is probably to be accounted for by some peculiarity of this chromosome, either by the relatively small number of genes that it contains, or else because the action of the genes is such that their absence or doubling produces no serious results. The fact that no flies have been found in which one chromosome II or III is absent, or in triplicate, must be supposed to be due to the fatal effect of such an absence or addition on the development of the individual. While this statement cannot be proved directly, it can be made highly probable by the results obtained when triploid *Drosophilas* are bred. Owing to the presence of three chromosomes of each kind in the triploid there are many possible kinds of eggs that are expected,[1] and Bridges, who discovered these triploids, and who has studied the problems of inheritance that they present, has found no flies triploid for II or III separately but only for II and III simultaneously, although such combinations must be produced at fertilization.

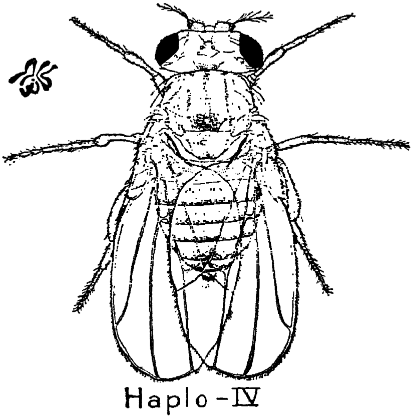

FIG. 12.—A haplo-IV fly. In the upper left-hand corner the group of chromosomes lacking one IV chromosome is represented.

In this connection it is important to note that among the offspring of these triploids there are individuals showing peculiarities of both sexes. These have been shown to arise when certain numerical combinations of autosomes and sex chromosomes are present. Since this condition has an important bearing on an understanding of the production of male and female, some of their characteristics may be described. The intersexes have three sets of autosomes and two *X* chromosomes ($3A-2X$). The difference between them and the normal sexes is obviously due not to the number of the *X*'s present (because these intersexes have two *X*'s as has the normal female) but to the balance between the autosomes and the *X*'s. Bridges has expressed this as follows:

> Both sexes are due to the simultaneous action of the two opposed sets of genes, one set tending to produce the characters called female and the other to produce the characters called male. These two sets of genes are not equally effective, for in the complement as a whole the female tendency genes outweigh the male tendency genes and the diploid (or triploid) form is a female. When the relative number of the

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[1] It has been shown by Belling in triploid cannas that the three chromosomes of each kind come together at the maturation period and segregate according to the non-disjunctional scheme. The resulting gametes contain all combinations of diplo- and haplo-chromosomes. Similar results have been recorded in mulberries by Osawa and in the evening primrose by Geerts (1909, 1911) and in *Datura* by Belling and Blakeslee (1922).

female tendency genes is lowered by the absence of one X, the male tendency genes outweigh the female and the result is the normal haplo-X male. When the two sets of genes are acting in a ratio between these two extremes, as in the ratio of $2X:3$ sets autosomes, the result is a sex intermediate—the intersex.

The intersexes are of two somatic types, one more like the female, one more like the male. The type that is more like the male seems to be due to the absence of one chromosome IV, while in the female type all three IV's are present.

There are two other distinct types of *Drosophila* with aberrant chromosome relations. One of them is called a supermale because he has three sets of autosomes and only one X. He differs from the normal male in several respects, and is sterile. The other type of individual is called a superfemale and has two sets of autosomes and three X's. These females are identical with those in non-disjunction strains that occasionally survive. They are weak, abnormal individuals, and are sterile.

How far these results with *Drosophila* are comparable with another extensive series of intersexes that Goldschmidt has produced by crossing different species or varieties of the gypsy moth is not quite clear. Goldschmidt has interpreted them as due to the relatively different values of the male and the female producing sex factors in the different races of these moths. In principle this amounts to nearly the same thing as changing the proportions of the chromosomes in the same species, as shown in the intersexes of the *Drosophila.* Goldschmidt's method of formulation, however, is quite different. He represents the factors for the male as *mm*, and he interprets each *m* as present in opposite members of the same pair of chromosomes. All the spermatazoa and half the eggs come to contain one *m*. The female factors are represented by *FF*, and are transmitted through the egg alone. At one time these were represented as cytoplasmic factors, but later as carried by the W chromosome. On the WZ–ZZ formulation, the W factor (W chromosome) is confined to the female line, but since on Goldschmidt's interpretation its influence is transmitted to both kinds of eggs, he is obliged to postulate that its effects are produced before maturation, i.e., before it is eliminated from the egg that becomes ZZ after fertilization. It may seem doubtful whether there is any need for these rather involved interpretations. The work of Doncaster, Federley, and Meisenheimer suggests that the situation in the *Lepidoptera* is not very different from that in *Drosophila.*

Returning to the problems connected with a change in the number of the chromosomes by the addition of a chromosome to the series, it is important to realize that such a procedure is one that cannot give rise to a permanent chromosome complex, although through such a beginning a pair of chromosomes may later result. Theoretically at least this might explain the addition of a new pair. The evidence as to the effects produced by the presence of one triplo-group is rather meager at present, but so far as it goes it appears that such an addition is apt to give a less viable individual than one that is an improvement on the typical form. Unless such a triplo-group produced a decided advantage, it would be quickly eliminated, since it is an unstable condition at reduction. Even though it should become stabilized by leading to a new chromosome pair, it is still not apparent that this doubling might not still further upset the normal development or structure or physiological properties of the organism.

On the other hand, the sudden doubling of all the chromosomes to produce a tetraploid individual appears to be a more successful means of affecting the chromosome relations, and the discovery both in nature and in cultivated plants of instances of closely similar (related) species having chromosome numbers as 1:2 has suggested to several geneticists the possibility that by means of doubling new types may have arisen in nature as they are supposed to do at times under cultivation. There are some physiological and cytological questions connected with the sudden doubling of the number of chromosomes that cannot be discussed here, and there are also some genetic questions of unusual interest. Two of these questions may be stated. First, the entire relation of the inheritance of recessive versus dominant characters is involved, for there are now four identical loci of each kind; a change in one of them to a recessive will give in F_2 not the usual Mendelian 3:1 ratio but a ratio of 35:1. Second, since there are four like chromosomes, several possibilities arise as to their mode of conjugation in pairs or otherwise. Perhaps the most general problem connected with the tetraploid condition is the opportunity it seems to offer for doubling permanently the entire number of hereditary factors. The possibility frees us from the discouraging conclusion that the number of genes has not increased since the beginning of cellular life, and may have even steadily decreased since that period!

There are a few other changes in genetic behavior that have been traced to translocations of pieces of one chromosome on to another chromosome, leading to duplications of genes that affect the genetic behavior of such flies in very exceptional ways. As yet, these results rest, for the most part, on deductions from the genetic behavior, and cannot with certainty be identified by an examination of the chromosomes themselves. There can be little doubt from the genetic behavior that such losses and additions take place, but the pieces involved may still be too small to be detected by modern cytological methods except under very favorable conditions. Two of the most interesting cases of a deficiency may be given by way of illustration.

There is a race of *Drosophila* in which, as Bridges has shown, the end of chromosome II is broken off and is joined near to the end of chromosome III. The length of the piece is eight units of map distance. The loci in this translocated piece contain the allelomorphs of plexus, brown, blistered, morula, speck, balloon, purploid, and lethal II*a*. Any fly that has one end of chromosome II absent dies, even although it has in the other chromosome II a normal end containing one series of normal genes. But the fly lives if the piece missing from chromosome II is attached to chromosome III. It is also significant in this connection that throughout this section of eight units there is no crossing-over in a female, if one of her chromosomes has this deficient end.

In another race of *Drosophila* known as Notch there is a region of four units length near the left end of the *X* chromosomes, where, as Mohr has shown, there is no crossing-over, and which normally contains the genes from white to abnormal inclusive. In a female one of whose *X* chromosomes contains a recessive gene whose locus is in this region, and whose other *X* is deficient for this region, the recessive character appears as a dominant, for the same cause that any sex-linked character appears as though it were a dominant in a male that has only one *X* chromosome.

These recent discoveries relating to chromosome changes have a bearing on an interpretation of Mendelian characters that has had a rather general vogue. It is known as the presence-and-absence theory. It originated with Bateson and Punnett, and is not a part of Mendel's original contribution. Mendel represented the symbol for a pair of characters by a large and a small letter (*Aa*). In the presence-and-absence theory that also makes use of a capital and a small letter, the recessive gene, represented by a small letter, is interpreted as a loss. As a rule, this is merely a symbolic statement, applying now to the character, now to the gene. By a sort of indirect implication a dominant character is looked upon as something added to the original type. This idea suggested itself from a frequently observed peculiarity of some recessive characters that very commonly involve a loss of something from the known or inferred parent type. For example, albinism is by definition an absence of a pigment producing property of the organism. On the other hand, it is to be remembered that there are white races that are dominant over the wild or colored type; the white leghorn race of poultry is such a case. An attempt has been made to bring such a dominant relation into line with the absence hypothesis by assuming that a dominant factor is present in the wild type that suppresses white plumage, and by its loss the color-producing factors still present become effective. Logically, this clears the situation of ambiguity, but has little else to recommend it.

There are many facts that can scarcely be forced into the interpretation of presence and absence. A great number of recessive characters might be said to add something to the wild type—or could be so interpreted—such as melanic forms that are as obviously additions as white is an absence. There are dominant characters that are just as plainly losses of character as are the stock examples of such recessives; for example, the loss of one series of finger bones in brachydactyly. Finally, there are the cases of multiple allelomorphs—at least those that are known to have originated independently from the wild type—where ten absences would be required to apply the hypothesis!

While it is of little importance to the Mendelian theory of heredity how we choose to picture to ourselves the nature of the changes in the germ material that affect the development in such a way that something different emerges, there is a small body of evidence that has a very immediate bearing on this question if it is no longer regarded as a matter of symbols but as an expression of the actual changes that take place in the germ material. For instance, addition of an extra chromosome in the evening primrose and in *Datura* and the loss or addition of a IV chromosome in *Drosophila* furnish an opportunity of studying how such additions or subtractions affect the characters of the individual. Here, then, where we can discuss specific instances, the results lend no support to the "presence-and-absence" interpretation of genes, in the sense that an absent chromosome produces an effect different in principle from the presence of a supernumerary one.

XIII. CYTOPLASMIC INHERITANCE

In addition to the large number of characters whose inheritance is compatible with the view that their forerunners are carried in the chromosomes, there are a very few instances where certain characters are transmitted only through the egg, and not by the sperm. Hence it has been inferred that there may be self-perpetuating bodies in the cytoplasm that are the representatives of these characters. Since there is no known clean-cut segregation process in the cytoplasm, it is not to be expected that cytoplasmic inheritance would be the same as that of characters represented by genes in the chromosomes. There is in fact no resemblance.

The only case of inheritance through the cytoplasm that is clearly established is the green color of the higher plants due to chlorophyll bodies that are known to be self-perpetuating, and to be distributed irregularly from cell to cell at the division periods. There is nothing in such cases as these that is in any way to be interpreted as in opposition to Mendelian inheritance. It is in fact surprising that so few instances have been found that are referable to the cytoplasm or to self-perpetuating bodies, or other inclusions that occur in the protoplasm, since there is an ever increasing body of information relating to cytoplasmic inclusions.

While genetics is not directly concerned with the question of the relation of the chromosomes to the cytoplasm—these phenomena belonging to the developmental aspect of biology—nevertheless, since there has been some confusion in the recent literature concerning these relations, it may be expedient to state the case briefly.

First, we may set aside all instances where the inheritance is due to self-perpetuating bodies in the cytoplasm. They present no difficulties to the genetic theory. In the second place, the few known instances of maternal inheritance, so called, but misnamed, are in harmony with the chromosome theory. Since the egg when it is fully formed has developed, as have all the cells of the body, in the presence of the entire genetic complex, it is obvious that if the egg has specific cell characteristics these would manifest themselves. It is quite reasonable to suppose that in addition to the visible characteristics of the egg (such as pigment) there may be other characteristic features, peculiar to the variety or species, that manifest themselves as soon as the developmental process begins. Such processes as the rate of cleavage and the type of cleavage, or even gastrulation, have been shown to be characteristics that have been impressed on the egg during its growth in the ovary. This has been proved in two ways. If eggs of one species or variety are fertilized by sperm of another species or variety, in which the rate of cleavage, for example, is different, it has been found that the rate of cleavage is that characteristic of the mother's species, and not that of the father's. In the second place, if, under such conditions, the eggs of a heterozygous individual be examined, it will be found that they all show the cytoplasmic characteristics of the dominant type even although after the polar bodies are eliminated one-half of the eggs have lost the dominant factors (by their extrusion into the polar body). The influence of the dominant factors remains in the egg, as far as its first phases are concerned. If, however, such influences should persist into the late developmental stages, then all heterozygotes would behave in inheritance as dominants, and a Mendelian ratio of 3 : 1 would not be observable. In fact, however, the evidence is quite clear, and shows that the presence of dominant factors (genes) in the egg before maturation has no effect at all in the later development of the recessive features in such an egg if it is fertilized by a recessive-bearing sperm. Were it not for this general relation, the theory of inheritance would be very different from what it is today.

XIV. RELATION OF GENES TO CYTOPLASM

Although, as stated above, the genetic problems in a strict sense are concerned with the shuffling of the genes between generations, nevertheless the genetic work has thrown some light on certain aspects of the relations of the genes to the cytoplasm, which is one of the problems of embryonic development. Certain interpretations of this relation have been advanced that are now known to be erroneous, and genetics has had a share in exposing their fallaciousness. Even before the relation of Mendelian factors to the chromosomes had been generally recognized, Boveri (1903) had suggested that the cytoplasm is concerned with the transmission of the "fundamental" characters of the species, and that the chromosomes carry only those factors that involve the details. The same idea has been repeated by several later writers. It had its origin, probably, in the fact that most of the visible changes that take place during the early stages of development appear to be concerned with changes in the cytoplasm in different parts of the egg, that seem to be related to the relative distribution of the cytoplasm of the fertilized egg, or at least to follow the axial and bilateral distribution of the cytoplasmic material. Such relations as right and left, anterior and posterior, and the spacial distribution of the parts of the embryo in relation to the early established planes of symmetry and localization of organs along axial lines, seem to be laid down at the beginning of development. All this takes place in the presence of the whole genetic complex, that might seem, then, to have no discriminating influence on the regulation of these processes.

It is also true that by far the greatest number of characteristics that students of Mendelian inheritance have concerned themselves with relate to superficial differences, such as shades of color or slight differences in the length or breadth of characters that are relatively unimportant for the individual. The explanation of this procedure is obvious enough, for it is just these slight differences that do not interfere with the survival of those individuals that are necessary to the geneticists. But there is no line that can be drawn between these trivial differences and those that are more significant, and fundamental. Even such a fundamental property as symmetry has been shown to depend on a single Mendelian gene as when the bilateral flower of the snapdragon changes to a peloric flower with radial symmetry. So far as Mendelian factors are concerned, the evidence is quite sufficient to show the erroneousness of the view that Mendelian genes are concerned only with trifling differences.

That certain fundamental characters are determined in the cytoplasm of the egg no one will question, but what takes place here may have first been determined by the genetic complex. That the genes brought in by the sperm may not at once change the cytoplasm is not surprising. The surprise is rather that the early influence of the chromosomes on the cytoplasm, when it occurs, is so quickly lost, as seen whenever a heterozygous egg produces a recessive individual if fertilized by a recessive-bearing sperm.

It is sometimes said that the cytoplasm must be as important as the chromosomes, since no development is known except in the presence of the cytoplasm, and by its activity. Whether the cytoplasm or the chromosome is or is not equally "important" is a matter that cannot be determined, and is of very little consequence. The statement is an example of obscurantism rather than of profundity. What genetics has so far discovered that bears on this relation can be briefly stated. It is this. All the examples of heredity that have been sufficiently worked out show that all adult characters and most embryonic ones (not even excepting those at the beginning of development which are given above) are accounted for by the known behavior of the chromosomes. In other words, they "follow" the chromosomes regardless of the source from which the protoplasm comes. An example may make this clearer. A female fly with pink eyes produces eggs, which, if fertilized by sperm from a red-eyed fly, give rise to offspring with red eyes. Conversely, a female with red eyes, fertilized by a male with pink eyes, also gives rise to offspring with red eyes. It has made no difference whether the cytoplasm of the egg came from the red- or the pink-eyed stock. The failure of the cytoplasm to influence the outcome is even better shown by back-crossing the F_1 (heterozygous) fly from either of the last two crosses to a recessive pink-eyed male. Half the offspring are red- and half pink-eyed. The latter are identical in eye color with flies both of whose parents were pink-eyed. Here the egg cytoplasm has been produced in the presence of the dominant gene in one of the chromosomes, but this has no effect on the eye color, if, after extrusion in the polar body, the red-producing genes are lost. It is clear that whatever the cytoplasm contributes to development is almost entirely under the influence of the genes carried by the chromosomes, and therefore may in a sense be said to be indifferent.

These statements are not intended to imply that the cytoplasm is a negligible element in the development of the organism. On the contrary, the developmental processes appear to be entirely dependent on it. How the genes in the chromosomes produce the effects in and through the cytoplasm we do not know. There is no evidence whatever to show that materials produced by the genes pass out and make the cytoplasm. For all we know to the contrary, most effects may be produced by chemical materials set free from the genes that affect the cytoplasm as long as they are produced. If, as appears to be the case, the cytoplasm grows and divides through its own activities, it is inherited just as definitely as are the genes, in the sense that it is transmitted from one generation to the next. The cytoplasm of the eggs of two mutants *may* be as different as are the genes that constitute the chromosome complex of the two mutants; but the cytoplasm in the two mutants *may*, so far as we know, be identical in so far as it changes in reference to whatever kind of genes are present when it develops. On the other hand, the cytoplasms of two types may be different in the sense that in some respects they are affected differently—if affected at all—by the genetic chromosome groups. These questions must be kept entirely free from predilections until we have found out more about the physiological processes that take place in the chromosomes and in the cytoplasm. Whatever the future has in store for us in these respects, the answer does not prejudice the present situation so far as the observed effects of the genes in heredity are concerned.

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